XIV. THE FORMATION OF A PEPTONE FROM CASEINOGEN BY THE PROLONGED ACTION OF DILUTE HYDROCHLORIC ACID IN THE COLD.

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The partial hydrolysis of proteins by strong acids in the cold was worked out very extensively by Emil Fischer, Abderhalden and their co-workers. As the result of the hydrolysis a number of well defined polypeptides were isolated. Methods were also devised for their separation.

We are not aware however that hydrolysis has been attempted by the prolonged action of weak acids, such for instance as hydrochloric acid in double the concentration in which it occurs in the gastric juice of the dog. The question arose whether hydrolysis took place at all in these conditions and if it did whether the products differed from those obtained by hydrolysis with strong acids.

For the purpose of testing these possibilities commercial caseinogen was left for three months in contact with 1 % HCl-solution. With the aid of the usual methods a product was separated, which, although not quite pure, proved to be a peptone. Although the amount of the substance available for the investigation was small it was possible to determine some of the amino-acids present in the preparation by using the excellent methods brought forward by van Slyke and Folin. As a result of this investigation it was found that the peptone obtained contained only a trace of tyrosine, whereas the peptones separated in this way are usually very rich in this amino-acid. This perhaps suggests that weak acids act on proteins differently from strong acids. A careful control of the commercial caseinogen was made to ascertain whether the product isolated was not present preformed in the commercial preparation, with entirely negative result. This control was performed because it seemed surprising that such weak acids as we had used were able to disintegrate the protein molecule to such an extent.

During this enquiry we had the opportunity to investigate the action of weak acids on the reaction of peptones with triketohydrindene hydrate and we have found that a drop of a decinormal solution of a mineral acid inhibits the reaction completely; the action of organic acids in a ten times higher concentration is the same.

Experimental.

1 kg. of commercial caseinogen was left in contact with 5 litres of a 1 % HCl-solution for three months. The suspension of caseinogen which had not been attacked was filtered off. The filtrate which had a deep brownish colour was acid and still contained free hydrochloric acid. The liquid which showed no sign of putrefaction gave a strong biuret reaction, but no Millon reaction. The solution was precipitated in the usual way with a 50 % solution of phosphotungstic acid. After 24 hours the precipitate formed was filtered off. The filtrate was still found to give a strong biuret reaction but was not investigated any farther.

The phosphotungstate obtained was decomposed in the usual way with baryta, and the filtrate evaporated *in vacuo* to a syrup which was dissolved in methyl alcohol and filtered into a large volume of absolute alcohol. The product separated was filtered off and washed with alcohol and ether. It was a white powder, fairly soluble in cold water and non-hygroscopic. Yield 14 grm.

The product contained a trace of ash, a trace of sulphur and no phosphorus. It contained 13·41 % N by Kjeldahl's method and 2·33 % of amino-nitrogen before hydrolysis.

The nature of the product was found to be that of a peptone as shown by the reactions described below:

I.	Millon	Trace. Traces of tyrosine.
II.	Diazo-reaction	+
III.	Triketohydrindene hydrate (Ninhydrin) ...	+
IV.	Folin's phenol-reagent	Trace.
V.	Xanthoproteic reaction	+
VI.	Biuret	Pink-violet.
VII.	Bromine	−
VIII.	Glyoxylic acid	+ [1]
IX.	Diazo-reaction after benzoylation of the product	−. No histidine.

[1] The glyoxylic acid reagent was prepared following Benedict [1909] by the action of magnesium on oxalic acid.

For the control 100 grm. of commercial caseinogen were extracted for one hour on the shaking machine with HCl of the same concentration as that mentioned above. In contrast to the previous one the suspension

filtered with great difficulty. The filtrate was colourless but slightly opalescent. The solution gave a precipitate with phosphotungstic acid which yielded on decomposition under the same conditions as above 0·4 g. of a white substance which proved to be mostly inorganic; it consisted of ammonia salts chiefly and gave no trace of a biuret reaction. One can conclude therefore that the peptone obtained was formed by the action of the 1 % HCl on caseinogen.

With a view to determining some of the amino-acids present in this peptone a complete hydrolysis was performed according to van Slyke's method. An estimation of tyrosine was done separately according to the method of Folin and Denis [1912], but only a trace of this amino-acid was found, due very likely to an impurity. An attempt was made to estimate tryptophane by the recently described colorimetric method of Herzfeld [1913], but the method proved in our case to be unsatisfactory as a decolorisation took place[1].

The result of total hydrolysis of the peptone.

Total nitrogen	13·35 %	Histidine-N.	0
Melanine-N.	0·15 %	Lysine-N.	(?) 5·23 %
Ammoniacal-N.	0·90 %	Cystine-N.	0·30 %
Total amino-N.	5·78 %	Total N. of the phosphotungstic acid filtrate	6·16 %
N. of the phosphotungstic acid ppt.	5·53 %		
Amino-N. of the phosphotungstic acid ppt.	2·78 %	Amino-N. of the phosphotungstic acid filtrate	1·92 %
Arginine-N.	0	Tyrosine-N.	0·09 %

The results of the hydrolysis seem to suggest that the only hexone base in the product is lysine, the nature of the monoamino-acids being still unknown.

The effect of acids on the reaction of the peptones with ninhydrin.

One drop of a decinormal solution of hydrochloric or sulphuric acid added to 10 cc. of the peptone solution completely inhibits the reaction, both with the peptone described above and also with Witte peptone. The same effect was obtained with one or two drops of a normal solution of acetic or lactic acid.

[1] The same decolorisation took place when we tried to estimate tryptophane in maize by this method.

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XV. THE GALACTOSIDES OF THE BRAIN. II. THE PREPARATION OF PHRENOSIN AND KERASIN BY THE PYRIDINE METHOD.

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The methods hitherto used for the preparation of the galactosides from brain may be divided into two groups. In one group of methods, which may be traced back to Couerbe's work, a separation of the galactosides from the phosphatides is effected by purely physical means, mainly by the help of such solvents as alcohol (Thudichum), glacial acetic acid (Koch), methyl alcohol-chloroform (Wörner and Thierfelder), etc. In the second group of methods the relative resistance of the galactosides to hydrolysis by baryta is used to separate them from the easily saponifiable phosphatides. The latter method which dates back to the work of Müller has been applied directly to the brain (Parkus) or to the mixture of galactosides and phosphatides etc., which is obtained by cooling an alcoholic extract of brain (Kossel and Freytag, Thierfelder, Fränkel, Lapworth).

The product obtained by both methods consists of a mixture of at least two galactosides, a fact which was first recognised by Thudichum. To the latter investigator belongs also the credit of having introduced the only available method for their separation, which may be termed a temperature fractionation method.

Both groups of methods possess one inherent disadvantage, which has considerably retarded the study of brain lipoids in general. Whilst allowing the separation of the galactosides, they make it most difficult or even impossible to isolate the saturated phosphatides which are present in brain. Indeed since Thudichum, all the subsequent investigators, until quite recently, limited their work to the preparation of the galactosides, neglecting altogether the phosphatide moiety of the original product[1].

[1] Thudichum isolated one of these phosphatides, called by him sphingomyelin, by means of its cadmium salt, and it has since been prepared by other methods by Rosenheim and Tebb. According to unpublished investigations of Rosenheim and Tebb, the cadmium method is altogether unsatisfactory.

Further, none of the authors who used the baryta method seem to have taken into consideration the fact that the galactosides are more or less hydrolysed by prolonged boiling with baryta in aqueous or alcoholic solution. Only quite recently it has been shown by Thierfelder and Loening [1912] that relatively short boiling with aqueous baryta does not split off galactose from these substances. It remains, however, a significant fact that only those observers who used the alcoholic baryta method found a high nitrogen percentage in their products. This fact suggests that their preparations contained certain amounts of the nitrogenous base sphingosine, set free by partial hydrolysis from the galactosides or from the phosphatide sphingomyelin. This possibility must be borne in mind, especially as the alcoholic baryta method has recently again come to the fore [Fränkel, 1911; Lapworth, 1913], without apparently any previous investigation of the action of alcoholic baryta on the native galactosides.

Principle of new method.

The difficulties and failures of the older methods used for the study of the brain lipoids may be traced back to the general use of alcohol as the primary solvent. The solubilities in alcohol of the various substances composing the complex lipoid mixture, as it occurs in brain, are such that their separation has baffled investigators ever since Vauquelin first applied this solvent to brain over 100 years ago. The problem becomes much simplified by discarding altogether the use of alcohol as a primary solvent, and by applying to the brain the principle of selective extraction as first suggested by Rosenheim [1906], and subsequently worked out by Rosenheim and Tebb [1909, 1910]. The method consists shortly in extracting brain, suitably dehydrated, at various temperatures with solvents which as far as possible dissolve out only one group of lipoids at a time. The method has so far led to the separation of the following lipoids:

(1) Cholesterol (by extraction with cold acetone[1]).
(2) Lecithin and kephalin (by extraction with ether or petrol ether).
(3) Galactosides (by extraction with cold pyridine).
(4) Sphingomyelin and sulphatide (by extraction with warm pyridine).

The method has already been described in detail in a previous communication in this journal [1913], as far as the preparation of the crude galactosides is concerned.

[1] Acetone has subsequently also been adopted by S. Fränkel as the primary solvent in his method for the preparation of brain lipoids. After the removal of the unsaturated phosphatides, lecithin and kephalin, this investigator again reverts to the use of alcohol and proposes to separate the mixture of galactosides, saturated phosphatides and sulphatides by the use of the old methyl alcoholic baryta method [1911].

Nomenclature.

In his "Proposals for the nomenclature of the lipoids" the author has already stated in this journal [1909] the reasons for discarding the names cerebrin, pseudo-cerebrin, cerebron and homo-cerebrin. It was proposed, following the suggestion of Posner and Gies [1906], to retain the original names phrenosin and kerasin, introduced by Thudichum, for the two principal galactosides isolated by him from brain.

This nomenclature is now generally accepted by most writers on the subject, and it seems only logical to extend it to the cleavage products of these substances. The author has therefore followed Posner and Gies [1906] in adopting the name "phrenosinic acid" for the fatty acid obtained on hydrolysis of phrenosin. The term "kerasinic acid" proposed by Thierfelder for the corresponding acid from kerasin seems however to be unnecessary since this acid has been identified by Rosenheim and by Levene with the longer known natural lignoceric acid.

The separation of phrenosin and kerasin.

The difficulty of separating the galactoside mixture into its constituents is probably due to several causes, the most important of which are the following: (1) the close resemblance in their chemical constitution (see later); (2) the close resemblance of their physical properties, such as solubility etc.; (3) their chemical inertness, which prevents the formation of any characteristic derivative suitable for their purification[1]; (4) the remarkable property of these lipoids to exist in the liquid crystalline condition (see next communication) must also be held responsible to a large degree for the difficulties of their separation, especially since O. Lehmann, the discoverer of this interesting state of matter, has clearly demonstrated the easy miscibility of liquid crystals; (5) the absence of any criterion by means of which the completeness of the separation can be judged. Melting point estimation and elementary analysis are practically useless and may even be misleading in this case as has been clearly demonstrated by the experience of Thierfelder and of Levene and Jacobs[2].

[1] I have, however, found that these galactosides, when treated in pyridine solution with benzoyl chloride, easily give benzoyl products. A method for the separation of phrenosin and kerasin might be based on the different solubilities of their benzoyl derivatives.

[2] In spite of the close agreement of many analyses of "cerebron," Thierfelder and Loening were able to show later [1910] that it contained an admixture of another galactoside. Levene and Jacobs [1912] were so much struck by the agreement of their analyses of "cerebrine" and kerasin, that they considered them for this, amongst other reasons, to be stereo-isomeric substances.

The following observations, made by Rosenheim and Tebb six years ago in connection with some other work on brain lipoids [1908], are therefore of considerable value for the separation of phrenosin and kerasin.

(1) Phrenosin (in pyridine solution) was found to be dextrorotatory, whilst kerasin is laevorotatory in the same solvent.

(2) The behaviour of the two substances under the polarising microscope is characteristically different. It allows the detection of phrenosin in presence of kerasin, and *vice versa*, by a simple test which will be described later as the selenite-plate test.

By the help of these two tests, the progress of the separation of the galactoside mixture may be controlled. For the actual separation the principle of temperature fractionation, introduced by Thudichum, was made use of. As, however, the previous experience of Rosenheim and Tebb [1908] had shown that by the use of alcohol, which was the only solvent employed by Thudichum, a complete removal of the phosphatides is practically impossible, I have successfully used acetone containing 10 % of water for the primary fractionation.

The mixture is thus separated into: (*a*) *the phrenosin fraction*, deposited on cooling the acetone solution, from 50° to 36°, and (*b*) *the kerasin fraction*, deposited from the clear decanted mother liquor of (*a*) on cooling from 28° to about 0°.

It can easily be demonstrated by the use of the selenite-plate test that the phrenosin fraction still contains kerasin, and that phrenosin is present in the kerasin fraction. Their complete separation cannot be effected by the repetition of the temperature fractionation process with any single solvent. A series of fractionation experiments was therefore carried out with various mixtures of solvents and the products tested by the selenite-plate test as well as by the polarimeter. The following procedure was finally adopted.

Phrenosin is obtained free from kerasin by recrystallisation of the phrenosin fraction from a glacial acetic acid-chloroform mixture (3 : 2) at 37°. After the third recrystallisation from this solvent, it is usually found that the filtrate from the deposit formed at 37° no longer deposits kerasin when cooled to room temperature. The last traces of phosphorus are also removed by this solvent.

The selenite-plate test, however, still reveals at this stage the presence of small quantities of kerasin, which are removed by repeated temperature fractionation from a mixture of acetone-chloroform (3 : 2). The last filtrate from the deposit at 37° must remain perfectly clear on cooling to room temperature.

The product is finally once more recrystallised from acetone containing 10 per cent. pyridine.

Kerasin is obtained free from phrenosin in a similar way from the kerasin fraction by using the same solvents. In this case, however, the superficial criterion of the removal of phrenosin is given by the fact that the solution remains perfectly clear at 37° and only begins to deposit when cooled to 28°. The final product, recrystallised again from acetone-pyridine, should be laevorotatory, and when examined under the polarising microscope, show the complete absence of phrenosin.

A comparison of the substances thus obtained with phrenosin and kerasin prepared previously by Rosenheim and Tebb [1908] according to Thudichum's original method, showed a close agreement in elementary composition, melting point, optical activity, appearance under the polarising microscope and in their hydrolytic cleavage products. As far as our present knowledge admits, the identity of phrenosin and kerasin prepared by Thudichum's method and by the new pyridine method may be considered as proved.

Experimental.

The crude galactoside mixture, the preparation of which has already been described in detail in Part I of this series of communications [1913], was used for the isolation of the substances to be described. After two recrystallisations from an alcohol-chloroform mixture, it still contained a small percentage (0·08 %) of phosphorus. As the selenite-plate test was found to be most useful for controlling the progress of the separation of this mixture it may be described next.

The selenite-plate test. Both phrenosin and kerasin separate from a warm 10 % solution in pyridine on gradual cooling in the form of spherocrystals, possessing approximately the same refractive index as the solvent. They are therefore scarcely visible under the microscope in ordinary light. In polarised light, with crossed nicols, they stand out bright on the black background and show well-defined crosses. If a selenite-plate (Red I) is placed below the stage and immediately above the polariser in such a way that its axis lies diagonally to the planes of polarisation of the crossed nicols, a characteristic difference between phrenosin and kerasin will be noticed at once. On the red background the spherocrystals appear to be divided into quadrants, of which two opposite ones show the addition colour, blue, whilst the two others show the subtraction colour, yellow. But, while the spherocrystals of phrenosin, under the above conditions, show the blue colour in

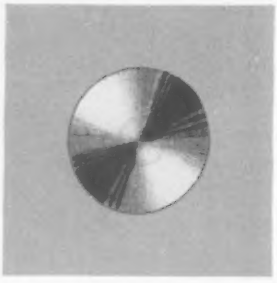

Fig. 1
Spherocrystal of phrenosin

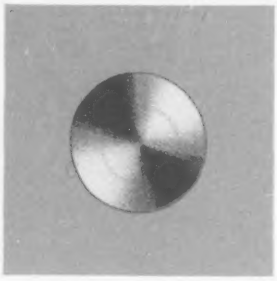

Fig. 2
Spherocrystal of kerasin

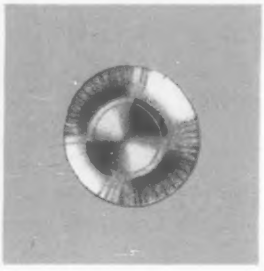

Fig. 3
Spherocrystal of a phrenosin-kerasin mixture

Reproduced from colour photographs (Lumière's process), taken in polarised light with a Selenite plate (Red I). The arrow indicates the direction of the axis of the Selenite plate.

the upper right and lower left quadrants (Fig. 1), the reverse is the case when the spherocrystals of kerasin are observed under exactly the same conditions (Fig. 2). Or, in other words, to use the nomenclature proposed by Göthlin [1913], the paragonal quadrants of the phrenosin spherocrystals show the addition colour, and the epigonal quadrants the subtraction colour, whilst the kerasin spherocrystals show the reverse behaviour. If we accept the current views according to which a spherocrystal is composed of radially arranged prisms, we may say that a spherocrystal of phrenosin is positively anisotropic, i.e. it is composed of optically positive prisms which are radially arranged. Kerasin on the other hand yields negatively anisotropic spherocrystals under the same conditions.

By means of this simple physical test, it is possible to establish in a very small sample the complete removal of kerasin from a phrenosin preparation, and *vice versa*.

It was found that from a galactoside mixture (i.e. either from any impure phrenosin or kerasin fraction, or from an artificial mixture), spherocrystals of both kinds are formed. They usually appear at first as separate crystals, varying in size from 0·05 to 0·5 mm. If the preparation is left for some hours, however, a kerasin crystal may deposit round a spherocrystal of phrenosin, or *vice versa*. A typical crystal of this kind, obtained from a phrenosin fraction, is reproduced from a colour photograph in Fig. 3. The limit of the sensitiveness of this test has not yet been established quantitatively. That it is, however, sufficiently sensitive for practical purposes, follows from the fact that a laevorotatory kerasin fraction, which was found to be free from phrenosin by the selenite-plate test, was also found on hydrolysis to be free from phrenosinic acid, the typical fatty acid of phrenosin.

In order to economise the valuable material, the test was carried out by dissolving a very small quantity (8–10 mg.) of the substance in a small test tube (0·5 × 4 cm.) by adding two drops of pyridine and warming to about 37°. A drop of the solution was transferred by means of a warm capillary pipette to a warmed slide, covered and allowed to cool gradually. A layer of spherocrystals forms at first round the edges of the coverslip, thus preventing further evaporation of the solvent, a fact which makes it possible to preserve the preparation for reference.

Fractionation of the galactoside mixture from acetone.

Previous work of Rosenheim and Tebb [1907] in connection with brain lipoids had demonstrated the advantage of acetone as a solvent for galactosides[1]. I have since found by a series of systematic experiments (omitted to save space), that the solvent power of acetone for galactosides is greatly increased if it contains 10 % of water.

The finely powdered galactoside mixture was therefore treated in portions of 50 g. with 3500 cc. of 90 % acetone in a water-bath kept at 56°. Only one extract was made and the insoluble part (about 15 %) has not yet been further examined. The clear acetone solution was allowed to cool in an incubator at 37°. After 16–20 hours a crystalline deposit, partially adhering to the sides of the glass vessel (phrenosin fraction), had formed, from which the supernatant fluid was easily decanted through a filter warmed to 37°. The filtrate was allowed to stand for 24 hours or longer in an ice chest, and the bulky gelatinous precipitate considered as the kerasin fraction.

The precipitates were filtered under pressure, washed with acetone and dried *in vacuo*. The kerasin fraction amounted to about 50 % of the phrenosin fraction, but the actual amount of kerasin contained in it is naturally much smaller, as only a rough separation is effected by this treatment.

Phrenosin.

32·5 g. of the phrenosin fraction were recrystallised from ten volumes of glacial acetic acid-chloroform (3 : 2). The finely powdered substance was first dissolved in 120 cc. of chloroform at about 60°, and to the solution was added 180 cc. of acetic acid previously warmed to the same temperature.

The clear solution was kept in an incubator at 37° over night, the deposit filtered and washed at 37° with the acetic acid-chloroform mixture. The moist precipitate was again dissolved in 200 cc. of the solvent and the solution treated in the same way. (The mother liquors of these two recrystallisations deposited on cooling to room temperature a considerable amount (6·64 g.) of a gelatinous precipitate, which was worked up with the kerasin fraction.)

The product deposited at 37° was granular and easy to filter. When recrystallised a third time in the same way, the mother liquor no longer

[1] This solvent has since been used for the same purpose by L. Smith and Mair [1910], and by Thierfelder and Loening [1912].

deposited on cooling to room temperature. After drying *in vacuo* the product weighed 20·1 g.

The selenite-plate test at this stage reveals only a small quantity of kerasin. A phosphorus estimation (Neumann's method) was intended, but 0·5532 g. of the substance carried through the process gave no trace of precipitate with the molybdic acid reagent, and the substance was therefore to be considered as phosphorus-free.

As the acetic acid-chloroform mixture did not seem to effect any further separation of kerasin, the product was now recrystallised from 100 volumes of acetone-chloroform (3 : 2). 19 g. of the product were first dissolved in 760 cc. chloroform and 1140 cc. warm acetone were added. The granular deposit, filtered at 37°, was twice more recrystallised in the same way. It then appeared to be perfectly uniform, as tested by the selenite plate.

In order to test its homogeneity, however, the product was separated into two fractions by using acetone-pyridine (equal parts) as a solvent. 13 g. were dissolved at 45° in 130 cc. pyridine and 130 cc. warm acetone were added. After keeping the solution for two hours at 35°, the deposit formed at this temperature was filtered off, and the solution cooled to room temperature. A further precipitate formed. The two fractions will be designated as phrenosin I a (6·5 g.) and phrenosin I b (5·2 g.).

From 30 g. phrenosin fraction of a different preparation there were obtained: 5·3 g. phrenosin II a and 4·7 g. phrenosin II b.

All the preparations, before being subjected to detailed investigation, were finally recrystallised from a large volume of acetone containing 10 per cent. of water.

All the preparations gave identical results on elementary analysis. They showed the same behaviour on melting and possessed the same optical activity. Further they gave on hydrolysis qualitatively and quantitatively the same products. These facts furnish strong evidence that a uniform substance had been isolated.

The product was further compared with phrenosin prepared according to Thudichum's original method. Thudichum fractionated his "white matter" from absolute alcohol at 37° and at room temperature. The fraction obtained at 37° serves for the preparation of phrenosin. Before or after the fractionation process, the product is treated with an ammoniacal alcoholic lead acetate solution in order to remove substances of the "cerebrin acid" type, which form insoluble lead salts. Thudichum's directions were closely followed, but as he had experienced considerable difficulty in obtaining his substance completely free from phosphorus, it was found advisable, in the light of later

experience, to use other solvents (glacial acetic acid, chloroform) as well as alcohol after the primary fractionation process. The lead treatment was carried out at the end of the fractionation process. Only traces of the "cerebrin acids" were found to be present. 90 g. "white matter," free from cholesterol, yielded finally 10 g. phrenosin. The product was free from phosphorus and sulphur and agreed in every detail with phrenosin prepared by the new pyridine method.

Kerasin.

From the kerasin fraction (see above), kerasin was separated in the following way: portions of 10 g. were dissolved in 40 cc. chloroform at 50° and to the solution were added 60 cc. glacial acetic acid previously warmed to about 60°. The solution remained clear until the temperature had fallen to 40° and was then kept in an incubator at 37°. A granular white layer, mainly phrenosin, collected on the surface and was filtered off at 37°. The filtrate began to deposit on cooling to 26° and solidified finally to a gelatinous mass. After filtering without pressure, and washing with the chloroform acetic acid mixture, the product was suspended in acetone and could be filtered under pressure. The product, when dry, weighed only 5 g. and was again recrystallised from 50 cc. of the chloroform acetic acid mixture as above described. Only 0·5 g. came down at 37°, the main quantity depositing at room temperature. The process was repeated twice more, when it was found that the solution no longer deposited at 37°, even when kept at that temperature for many hours. At this stage the selenite-plate test showed that the substance consisted practically only of kerasin, only a few isolated spherocrystals of phrenosin appearing after some hours.

Further purification was obtained by continuing the recrystallisation from 20 volumes of an acetone-pyridine mixture (equal parts). Three grams were dissolved in 30 cc. pyridine and 30 cc. acetone warmed to 45° were added. Only a faint cloud appeared on cooling to 37° in the incubator. This small deposit of phrenosin weighed 0·01 g. when filtered and dried *in vacuo*. The main filtrate began to deposit at 28° and was allowed to cool to room temperature before filtration. This process was repeated and the product finally recrystallised from a large volume of 90 % acetone containing 2 % pyridine.

The kerasin preparations were considered pure when only kerasin spherocrystals were found by the selenite-plate test and when their optical activity was not less than $[\alpha]_D = -2°$.

The separation by these purely physical means necessarily entails large losses, but leads finally to a pure product, a fact which was confirmed by the investigation of the hydrolytic cleavage products. From 10 g. of the original kerasin fraction, there were obtained on the average 1·36 g. kerasin (from 10–15 %).

The physical appearance of kerasin when depositing from its solutions is characteristically different from that of phrenosin. Whilst the latter usually deposits in loose granular masses, kerasin forms a coherent jelly which contracts on shaking. When filtered and dried *in vacuo*, it can easily be powdered, but when allowed to dry in air it forms a translucent white wax-like substance, thus fully justifying the name given to it by Thudichum.

Kerasin was also prepared according to Thudichum's method from the kerasin fraction of "white matter" (see above). It was subjected to the lead and cadmium treatment as described by Thudichum. It was found that only minute traces of substances precipitable by these reagents were present, no doubt owing to the fact that mixtures of solvents (see above) were used for recrystallisation instead of alcohol alone. The observation of Thudichum with regard to the change of solubility in alcohol as purification proceeds was fully confirmed. A product which contains only traces of phrenosin begins to deposit kerasin even at 37°, while originally, in presence of a large proportion of phrenosin, it only deposits below 28°.

From 90 g. cholesterol-free "white matter," there were obtained finally 5 g. kerasin.

The product was free from phosphorus and sulphur and agreed in its composition and all its properties with kerasin prepared by the pyridine method.

The results of the elementary analysis, the optical activity and hydrolytic cleavage products of phrenosin and kerasin will be described in a subsequent publication.

The expenses of this research have been defrayed from a grant from the Government Grant Committee of the Royal Society.

Summary.

1. The galactoside mixture obtained by extraction of brain with cold pyridine can be separated into a phrenosin and a kerasin fraction by temperature fractionation from acetone.

2. Phrenosin is obtained by recrystallisation of the phrenosin fraction

at 37° from chloroform-glacial acetic acid mixture, followed by recrystallisation from a chloroform-acetone mixture.

3. Kerasin is obtained from the kerasin fraction by recrystallisation at room temperature from chloroform-glacial acetic acid mixture, followed by a pyridine-acetone mixture.

4. A test (the selenite-plate test) is described, by means of which the progress of purification is controlled.

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XVI. THE GALACTOSIDES OF THE BRAIN. III. LIQUID CRYSTALS AND THE MELTING POINT OF PHRENOSIN.

By OTTO ROSENHEIM.

From the Physiological Laboratory, King's College, London.

(*Received February 24th, 1914.*)

The remarkable ease with which the brain galactosides are transformed into the liquid-crystalline state, was first observed by Rosenheim and Tebb [1908] in connection with some other work on the brain lipoids and has since been confirmed by Prof. O. Lehmann, who kindly examined phrenosin and kerasin (as well as sphingomyelin) prepared by the author. This phenomenon offers an explanation for the discrepant statements with regard to the melting points of these substances recorded in the literature. It further led to the discovery of the conditions under which phrenosin can be obtained in true crystalline form, which in its turn satisfactorily explains a hitherto unexplained property of phrenosin, for which the provisional term "Umlagerung" was used by Thierfelder and Wörner [1900]. A short description of my observations in this direction seems therefore justified.

(1) Liquid crystals, true crystals and myelin forms of phrenosin.

Phrenosin as usually obtained on cooling its solutions, consists of a white "crystalline" powder, which, however, does not possess any definite crystalline form in the usual sense. Under the microscope it is seen to consist of separate ball-shaped masses of approximately uniform size, which are only slightly anisotropic.

The formation of liquid crystals is best observed when a small quantity of the dry powder is carefully heated on a slide on Lehmann's polarisation microscope[1] [1910] until it is completely fused. In this condition it will be found to be completely isotropic, i.e. invisible in the dark field between crossed nicols. On allowing the slide to cool slightly a shower of separate anisotropic

[1] A very convenient electrical hot stage has been devised by Lorrain Smith and W. Mair [1910].

needle-shaped liquid crystals shoots out on the dark background (see Fig. 1). They may be best observed in the thinnest layers of the preparation and when pressure is exerted on the cover glass.

I made the further observation that phrenosin not only exists in the intermediary liquid-crystalline state when heated below its "clearing point," but also when it is allowed to settle out from its hot solutions in various solvents. This fact may also easily be demonstrated by means of Lehmann's microscope possessing a heating arrangement, using an alcohol solution of phrenosin. The liquid-crystalline globules which appear at first solidify on complete cooling to nodular masses, radially striated, thus forming the "crystalline" white powder as described above. The liquid-crystalline modification may even be observed macroscopically, if the dry substance is warmed with insufficient solvent. It then forms transparent globules which collect at the bottom of the flask and which were thought to be due to "decomposition" by the early investigators of brain lipoids.

It occurred to me that it might be possible to prevent the primary formation of the liquid crystals by some means and thus favour the formation of true crystals. After many unsuccessful attempts with various solvents under many conditions, it was found that true crystals were formed when a hot 2% solution of phrenosin in 85% alcohol was allowed to cool very gradually and without shaking, in an unsilvered Dewar flask. The flask was firmly plugged with cotton wool and kept in a water-bath at 75°. The temperature of the water-bath was allowed to fall and when the solution had reached a temperature of 65°, the first crystals appeared. Their quantity increased slowly and at 61° the whole solution was filled with a mass of glittering crystals, which were seen under the microscope to consist of thin, transparent, well defined plates, closely resembling cholesterol crystals (see Fig. 2). Highly purified phrenosin may thus be obtained completely crystallised. After filtration and drying it appears as silky flakes, its macroscopical appearance also resembling that of cholesterol. The substance may be recrystallised in the same way, but on allowing its solution to cool gradually in the ordinary way, the usual micro-crystalline powder (solidified liquid crystals) is obtained.

The air-dry crystals, when obtained from dilute alcohol, contain one molecule of water[1]. The water of crystallisation is not given up on drying *in vacuo* over concentrated H_2SO_4, but escapes on drying the substance at 105°.

[1] It is possible that the crystals obtained from absolute alcoholic or methyl alcoholic solution contain alcohol of crystallisation, but this question has not yet been experimentally investigated.

Fig. 1. Liquid Crystals of Phrenosin. ×90.

Fig. 2. Crystallised Phrenosin. ×50.

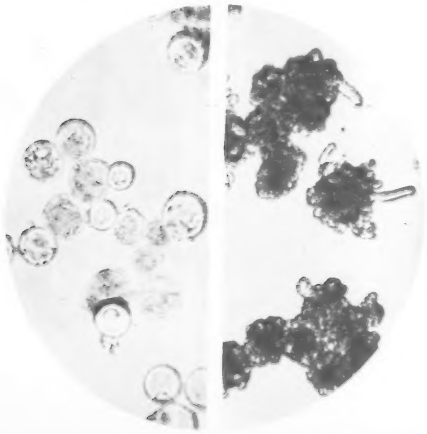

Fig. 3. *Left:* Phrenosin swelling up with water. *Right:* Early stage of Myelin forms. ×250.

0·4253 g. crystallised phrenosin, on drying in a toluene bath, lost 0·0100 g. = 2·35 % H_2O. Calc. for $C_{48}H_{93}O_9N + H_2O$: 2·13 % H_2O.

On combustion of the substance, dried at 105°, the following results were obtained:

0·1300 g.; 0·3305 g. CO_2; 0·1291 g. H_2O. 0·0960 g.; 1·7 cc. N at 17·5° and 722·5 mm.

		Calc. for $C_{48}H_{93}O_9N$
C	69·34	69·65
H	11·11	11·24
N	1·91	1·70

The fact that phrenosin may, under certain conditions, be obtained in the true crystalline condition offers an explanation for an interesting observation made by Thierfelder and Wörner [1900] many years ago. They found that when "cerebron" (= phrenosin) was kept at 50° suspended in 85 % alcohol or at 60° in methyl alcohol-chloroform [Thierfelder and Loening 1910], the previously "crystalline" product was gradually transformed into true crystals. This process was assumed to be due to an intra-molecular change (Umlagerung) but Thierfelder was at a loss to explain this "Umlagerung," which he showed was not due to the entrance of alkyl-groups into the molecule. I have repeated this experiment successfully with all my samples of pure phrenosin in the solidified liquid-crystalline condition and would suggest, in the light of the above observations, as an explanation of Thierfelder's "Umlagerung," that it consists simply in the gradual change of the solidified liquid-crystalline modification into the true crystalline condition at the temperature which is favourable for its formation[1].

Another characteristic property of phrenosin, which is related to the liquid-crystalline condition, consists in its tendency to give rise to myelin forms in the presence of water. If a fine suspension of phrenosin in water is gently warmed on a slide under the microscope, the small ball-shaped nodules swell up and are seen to be suddenly covered with spicules, which gradually develop into myelin forms (see Fig. 3). I may add the further observation that under the polarising microscope and a selenite plate, these peculiar forms behave exactly like the myelin forms obtainable from ammonium oleate or lecithin. In the paragonal position they show the subtraction colour (yellow) and in the epigonal position the addition colour (purple to blue).

[1] Phrenosin can only be obtained in the true crystalline state if certain conditions are observed, a fact which brings out the analogy of the brain galactosides to the vegetable glucosides, especially to those containing galactose like the digitalis glucosides. The latter have also so far only been separated by physical means, and digitonin, for instance, can only be obtained crystallised under certain conditions. Even then it may still contain the amorphous galactoside, gitonin, as has recently been shown by Windaus and Schneckenburger [1913].

The myelin forms appear on slight warming far below the boiling point of water, and indications of them may be seen even at 37°[1]. If a slide is kept at this temperature and then allowed to cool it will be seen that water has been taken up by the phrenosin powder, transforming it into transparent globules. Under the polarising microscope they are now strongly anisotropic, show the black cross of spherocrystals and behave with a selenite plate exactly like the spherocrystals of phrenosin as obtained from pyridine [see Rosenheim, 1914].

(2) The melting point (clearing point) of phrenosin.

The melting points recorded in the literature for phrenosin (pseudo-cerebrin, cerebrin, cerebron) show remarkable variations. Thudichum gave it at 176° and the subsequent statements vary from 170° (Parkus) to 212° (Thierfelder and Wörner). The last investigators state that the substance becomes "moist" at 130° and gradually covered with fine droplets, at 200° it becomes slightly yellowish, and fuses at 209° on slow heating, at 212° on rapid heating, to a clear yellowish fluid.

In my first observations on phrenosin (prepared according to Thudichum as well as by the pyridine method), I noticed a similar behaviour. On slow heating in a Thiele apparatus the samples soften between 130°–140°, shrink together and partially fuse between 170°–190°, and melt to a clear fluid at 205°–206°. On rapid heating the same substances melt, after the preliminary softening at 130°–140°, fairly sharply at 212°–215°. According to the rate of heating and the width of the tube, it is possible to obtain what appears as a melting point at any temperature between 170°–215°. Crystallised phrenosin behaves exactly like ordinary "crystalline" phrenosin.

An explanation for this peculiar behaviour was found in the above described discovery that this substance exists in the liquid-crystalline condition between the temperatures of 95° and about 210°. This fact may also be very conveniently demonstrated, without a polarising microscope, by the help of the arrangement recommended by Stoltzenberg [1911]. A thin glass rod is introduced into the melting point tube and the liquid-crystalline condition is recognised by pressure exerted on the glass rod. Under these conditions it was found that phrenosin enters into the liquid-crystalline

[1] G. F. Göthlin [1913] was unable to observe this fact, because he evidently made his observations in the absence of any heating arrangement. The myelin forms of pure phrenosin were first observed on my preparations in Prof. Aschoff's laboratory [see R. Kawamura 1911]. L. Smith and W. Mair [1910] have also made similar observations on the "cerebrosides" prepared by their method.

condition below 100° (approximately at 95°) and forms on pressure a perfectly clear viscous fluid at 130°, showing a final clearing point at 212°–215°. The liquid-crystalline phase evidently possesses a greater viscosity than the liquid-isotropic phase and the change from one to the other may be readily observed.

The discrepant statements about the melting point of phrenosin, which were quoted as evidence against the identity of the various preparations, are satisfactorily explained by the existence of the liquid-crystalline modification. Some observers evidently considered the substance as fused during the liquid-crystalline phase, whilst others took the temperature of formation of the isotropic-liquid phase as the melting point.

In view of the above observations there is no justification to speak of a "melting point" of phrenosin, the proper expression being the "clearing point," i.e. the temperature at which it changes from the liquid-crystalline anisotropic condition into the isotropic liquid-amorphous condition. [See also Lapworth, 1913.]

It may be mentioned here that kerasin behaves in every respect similarly to phrenosin. It gives rise to liquid crystals and to myelin forms, although the latter are not so readily produced as with phrenosin. Its clearing point is, however, much lower, namely 180°. Further I have so far been unable to obtain it in the truly crystallised condition like phrenosin. This fact may be connected with the constitution of the fatty acid (lignoceric acid) contained in kerasin, which, in distinction from phrenosinic acid, does not contain the hydroxyl group in its molecule. It may further be assumed that, in analogy to the derivatives of cholesterol, the ability of the galactosides to exist in the liquid-crystalline condition is mainly dependent on their basic constituent sphingosine, which resembles cholesterol in so far as it has the constitution of an unsaturated alcohol[1].

Prof. O. Lehmann, to whom I submitted samples of phrenosin and kerasin for more detailed investigation of their liquid-crystalline modification, has kindly given me permission to communicate the following abstract of his observations which he intends to publish in full elsewhere.

"The first of the two preparations of phrenosin received (labelled "crystallised") consists of thin transparent plates, approximately rectangular, and

[1] The property to form liquid crystals is not limited to the brain lipoids. I have found that "carnaubon" which was first obtained by Dunham and Jacobson [1910] from ox kidneys also possesses a liquid-crystalline phase. According to some preliminary experiments of the author the main fatty acid of carnaubon seems to be identical with lignoceric acid and carnaubon itself appears to be a mixture of a galactoside of the kerasin type with a phosphatide of the sphingomyelin type. The fact that these lipoids form mixed liquid crystals explains the difficulty of their separation.

slightly anisotropic; the second of small globules of fairly uniform size which show between crossed nicols the black cross of spherocrystals and which had probably been formed by solidification of globular myelin forms. The behaviour of both preparations is identical. On warming the dry substance it is suddenly transformed into the anisotropic liquid-crystalline modification and on further heating into the isotropic melt. The liquid crystals depositing from the latter do not show any tendency to run together, but they are deformed by pressure on the coverslip just like other liquid crystals. If the solid crystals are brought into water and gradually warmed, the change into the liquid-crystalline modification takes place below the boiling point of water. The liquid crystals assume the shape and structure of myelin forms. The ball-shaped solid structures are suddenly covered with radiary processes which, however, are not pointed but of uniform thickness and with rounded ends. On cooling, all the spicules suddenly contract to about one half of their size and their contents are transformed into an aggregate of fine plates of the solid modification. By repeated warming and cooling the extension and contraction of the rod-like myelin forms (liquid crystals) may be produced over and over again. We have to deal here with changes of form of a similar kind to those observed previously by Lehmann with protocatechuic acid [see Lehmann, 1911, p. 333]. Phrenosin forms with lecithin and ammonium oleate mixed liquid crystals in all proportions. Even with a high percentage of phrenosin the liquid crystals which separate out of dilute alcohol show the phenomenon of flowing together just as those of pure ammonium oleate (or lecithin).

By means of some aniline dyes the myelin forms of phrenosin may be artificially coloured, similarly to those of ammonium oleate. The phenomena are most striking, if the water in which the phrenosin granules are allowed to swell up contains just sufficient methylene blue to colour it faintly. The myelin forms, appearing on warming, will be seen to be stained intensely blue. On contraction by cooling, the blue colour changes into a reddish violet. If the processes are extended again to their former length by warming, the reddish violet colour just as suddenly changes into blue.

Kerasin behaves similarly to phrenosin, when heated in the dry state. At a certain temperature it suddenly passes into the liquid-crystalline modification, which changes on further heating into the isotropic melt. Warmed with water it swells up enormously, forming myelin forms which are only slightly more refractive than water. In consequence of their high water content they are of a slimy fluid consistency. With methylene blue they only stain very slightly, their size being reduced by the admixture of

the dye. On heating a few grains of the original substance with water to a higher temperature, it is transformed into the liquid-crystalline modification and then myelin forms appear which are distinctly anisotropic and easily stained. On cooling, the two kinds of myelin forms solidify, suddenly undergoing a contraction just like those of phrenosin. The solidified myelin forms are not brittle, but still soft and plastic. Kerasin forms with lecithin and ammonium oleate mixed liquid crystals in all proportions just like phrenosin."

The expenses of this research have been defrayed from a grant from the Government Grant Committee of the Royal Society.

Summary.

1. The galactosides phrenosin and kerasin exist in the liquid-crystalline condition at temperatures from below 100° up to 180° (kerasin) and 215° (phrenosin).

2. This fact explains the divergent statements with regard to the melting point of these substances. Instead of a melting point they possess a "clearing" point, i.e. a point at which the anisotropic liquid-crystalline phase changes into the isotropic liquid-amorphous phase.

3. They give rise to myelin forms on warming with water which possess the same optical properties as the myelin forms of lecithin, etc.

4. The conditions are described under which true crystals of phrenosin may be obtained.

5. An explanation is thereby afforded for Thierfelder's "Umlagerung" of phrenosin.

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XVII. REMARKS ON Dr SYMONS' "NOTE ON A MODIFICATION OF TEICHMANN'S TEST FOR BLOOD."

By WILLIAM DUNBAR SUTHERLAND
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(*Received February 25th, 1914.*)

On p. 596 of Vol. VII there appear some strictures on the spectrum test for blood, which we consider to be not so well-grounded as the writer may think they are.

We may at the outset mention that we base our conclusions on the results obtained by us from the examination of articles in 749 cases, mostly of murder, that have come to our laboratory. In all 1819 articles, which were, or were supposed to be, bloodstained, have been examined by us in these cases.

The technique adopted by us is that devised by Hankin, whose long experience of medico-legal work led him to believe that it is the simplest and at the same time the most reliable method of obtaining the characteristic spectrum of *cyanhæmochromogen*, even when one has but a spot of blood no larger than a pin-point before one, a belief that our results have fully confirmed.

Briefly stated, the spectrum is obtained as follows:

If the stain be on a hard surface such as glass, steel, wood or cement, a fragment of it is removed by means of a sharp knife, conveyed to a microscope slide, and treated with a drop of 10 % solution of cyanide of potassium. Any superfluous fluid is removed by means of bibulous paper, and the preparation is then treated with a drop or two of solution of sulphide of ammonium, covered and examined *at once* under the $\frac{1}{2}''$ objective.

If it be a soft fabric that is stained, a minute portion of the stain is removed, plunged into boiling water for a couple of seconds, and then treated as above.

If blood be present, somewhere in the preparation there will be seen a spot of colour, the tint of which varies from the faintest pink to cherry-red, according to the amount of pigment present. If this spot be small it is brought into focus under the $\frac{1}{6}''$ objective; if it be of fair size the low power will suffice. The eye-piece of the microscope is removed and the long arm of a Browning's hand spectroscope, modified by Zeiss, is inserted into the tube. Under the short arm of the spectroscope is held a piece of paper, to reflect sufficient light for the reading of the wave-length scale, and the absorption-bands of *cyanhæmochromogen* are then identified. In rare cases only one band may be seen, but in all cases where blood is present this method of proving the existence of its pigment in the material examined will be found to be successful: for even stains that have been more than half washed-out give the characteristic spectrum when so treated.

In the wet season it often happens that all that can be seen on a blood-stained fabric is a spot or two of mould, but so far we have had no difficulty either in identifying blood in such a case, or in determining its origin by means of the precipitin reaction.

An *old* stain may require to lie soaking in the cyanide solution for about a minute, before the ammonium sulphide is added to it. The advantage of using a 10 % solution of potassium cyanide instead of a weaker solution is that the weaker solution may cause difficulty by more rapidly giving rise to diffusion of the pigment throughout the preparation: naturally the more concentrated the pigment at one spot the easier the recognition of its spectrum. Hence Hankin's use of boiling water to fix the pigment and prevent its diffusion along the fibrils of the piece of stained fabric, when the potassium cyanide solution is applied to it.

The ammonium sulphide solution should be prepared thus: strong solution of ammonia is diluted with four times its volume of water, and then saturated with hydrogen sulphide gas. Of the resulting preparation two parts are added to one part of ordinary dilute ammonia solution.

For routine practice we find it most convenient to use a small test-tube filled with this solution for the day's work, as the solution kept ready rapidly decomposes.

By so treating our stains we have no need of using Teichmann's test: for no substance save blood when treated as detailed above gives the colour reaction *plus* the spectrum of *cyanhæmochromogen*.

As to the "corpuscle test"—which Dr Symons states "has been found of very doubtful value, and very often impracticable in the case of old stains"—we would remark that we have nearly always been able to find enough

erythrocytes in a material—even earth—in which the presence of blood has been proved by the spectrum, to be able to say whether that blood was mammalian or non-mammalian. *A fortiori* we have been able to say that the cells before us were erythrocytes. By "nearly always" we mean in 97 % of cases. Only four out of 125 bloodstained specimens of earth gave rise to difficulty in determining the nature of the erythrocytes present.

For the determination of erythrocytes the fragment of stained material is allowed to soak in Vibert's fluid for half an hour, then teased out and examined under the oil-immersion lens. It will be remembered that Vibert's fluid is composed of common salt 2 grams, mercuric chloride half a gram, and distilled water 100 cubic centimetres. Of all the fluids devised by microscopists for the treatment of bloodstains we find it to be on the whole the most satisfactory, being thus able to confirm Masson's high praise of its merits.

XVIII. AN IMPROVED HYDROGEN ELECTRODE.

By GEORGE STANLEY WALPOLE.

From the Wellcome Physiological Research Laboratories, Herne Hill, S.E.

(*Received March 3rd, 1914.*)

Further use of hydrogen electrode vessels of the type described [Walpole, 1913] has resulted in some modifications justified by the more accurate results obtained.

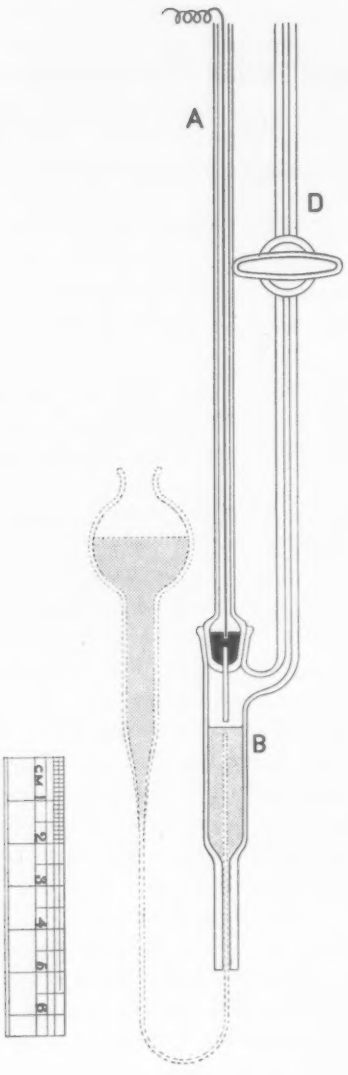
In the actual construction of the vessel the rubber stopper sometimes employed [1913, p. 411] was finally discarded, and a ground glass joint used instead where the glass tube *A*, carrying the electrode, fits into the outer tube *B*.

Both the electrode vessel, and the pipette necessary for refilling it when the fluid under investigation contains carbon dioxide, are made of hardest Jena glass, e.g. *Borosilicate glass, Jena* 59. 111. The slow rise of E.M.F. due to liberation of alkali from the glass is thereby avoided. The general dimensions are those given [1913, p. 415]. The description of a smaller electrode for electrometric titration [1913, p. 418] contains an error. Its capacity was 0·3 cc. not 3 cc. as stated.

In describing a determination it is assumed that the vessel is wet with the last fluid examined. Starting with a clean dry vessel the volume of fluid required is, naturally, smaller. The hydrogen supply is connected to *D*, and the air displaced. The tap on the T-piece [1913, p. 419] is shut, and, by means of the filling syringe, some of the liquid examined is drawn up into *B* and used to rinse it out. To do this *D* is shut and the electrode vessel tilted about the horizontal position.

For very careful work it is well to undo the ground glass joint and wet the surfaces with the experimental fluid before starting.

After emptying out the rinsing fluid by opening *D* and pushing home the piston of the filling apparatus, hydrogen is passed again for a second or two and the lower end of *B* dipped below the experimental fluid. The actual sample employed for the determination is then drawn up until the meniscus is just touching the blacked platinum point and the tap *D* is


A
D
B

closed. The rubber tube from the filling apparatus may now be detached. If the fluid contains no carbon dioxide the electrode vessel is placed at once in the trough containing saturated potassium chloride at 18°.

If carbon dioxide is present the hydrogen bubble must be passed backwards and forwards (five minutes is sufficient) till equilibrium is established, and the liquid then replaced by a fresh sample of experimental fluid from the pipette. To do this, the pipette, filled with the solution, is brought into the position shown in the diagram and in a short while the contents of *B* will be replaced. The hydrogen bubble is again passed to and fro for five minutes and the fluid replaced again. Three replacements are sufficient.

The size of the bubble will be found now to have increased. Connection to the filling apparatus and gentle suction with the lower end of the electrode vessel immersed in experimental fluid enables the adjustment of the meniscus to the platinum point to be made accurately. The electrode vessel is now placed in the potassium chloride trough without delay.

The pipette has a capacity of about 4 cc. and is half filled for each replacement. There is no need for a stopper of any kind at the bottom of the electrode vessel as capillary forces are quite sufficient to prevent egress of fluid or intake of air during manipulation. In those cases where blood-protein and similar solutions are examined the lowered surface tension produces difficulties which may be overcome by dipping the lower end of the filled electrode vessel in distilled water for a moment.

A P_H^+ determination of a carbon dioxide-sodium carbonate mixture containing 0·02 N NaCl, made by mixing 20 cc. 0·1 N HCl and 25 cc. 0·1 N Na_2CO_3 and diluting to 100 cc., with water gave the following results:

Time	E.M.F. reading against sat. KCl calomel electrode	
12.4	(Vessel introduced into KCl)	
12.5	0·6100	
12.10	0·6100	
12.30	0·6100	
2.10	0·6100	$P_H^+ = 6·22$

The experiment was commenced with the apparatus wet from a previous determination on another fluid; volume of fluid taken for examination 10 cc.

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XIX. THE ESTIMATION OF LACTOSE AND GLUCOSE BY THE COPPER-IODIDE METHOD.

By SYDNEY WILLIAM COLE.

From the Physiological Laboratory, Cambridge.

(*Received March 3rd, 1914.*)

Experience of the various methods that have been described for the estimation of the sugars by volumetric analysis has convinced me that the most exact one we have at present is that of Amos W. Peters [1912, 2]. Unfortunately he only gives the copper values for glucose. Being anxious to utilise the method for a research involving the estimation of lactose, I determined the copper values for this sugar and publish them now in the hope that they may be of some value to those engaged in milk analysis. I also give a description of the method, with some slight modifications that I have made, as I found the original papers rather difficult to follow.

Principle of the method.

A large volume of copper sulphate is prepared and its copper content accurately determined as described below. A given volume of the copper sulphate is treated with an alkaline tartrate solution and a known volume of the sugar and the mixture is heated under definite standardised conditions for a certain time. The mixture is filtered through asbestos and the copper in the filtrate determined by treatment with potassium iodide and titration of the iodine liberated by means of a solution of sodium thiosulphate. The liberation of the iodine is effected under standard conditions of temperature, dilution and acidity, and the thiosulphate is previously standardised against the copper sulphate under the same conditions.

Solutions required.

A. Copper sulphate. 69·278 g. of the purest crystalline salt, $CuSO_4$, $5H_2O$, are dissolved in distilled water and the volume made up to 1 litre. It is advisable to prepare a large volume of this stock solution.

B. Alkaline tartrate. 346 g. of Rochelle salt and 250 g. of pure potassium hydroxide are dissolved in water and the volume made up to 1 litre.

C. Sodium thiosulphate, N/5. 99·2 g. of the purest thiosulphate are dissolved in boiled out water and the volume made up to 1 litre with boiled out distilled water. A considerable volume can be prepared and allowed to stand about a week before use. It then acquires a value which is almost constant for some months. It should be stored in the dark.

D. Potassium iodide. Saturated solution; 100 g. of the solid treated with 70 cc. of hot distilled water and allowed to cool.

Standardisation of the thiosulphate and determination of the copper value of the stock solution.

Of the three methods given by Peters [1912, 1] the simplest is that of calculating the copper from the weight of $CuSO_4$, $5H_2O$ taken. I find that if Kahlbaum's purest copper sulphate "with certificate of analysis" be employed and allowance be made for the very small amount of impurity present as shown on the certificate, the results agree exactly with those obtained by the electrolytic method and by the "nitric acid-talcum" process of treating pure metallic copper. Consequently I shall only describe that method.

20 cc. of the copper sulphate solution are measured by means of a standard pipette into a 200 cc. Erlenmeyer flask, 40 cc. of distilled water and 20 cc. of strong acetic acid (33 %) are added, a thermometer is inserted and the mixture cooled or warmed to 20°. About 6·5 cc. of the saturated potassium iodide are run in, the thermometer being withdrawn and its stem washed with this solution. The iodine liberated is titrated at once with the thiosulphate. When approaching the end point 2 cc. of a 1 % solution of soluble starch are added and the titration completed. The colour changes to a chocolate-brown when very near the end point. This is best determined by the "spot test" method. Allow a drop of the thiosulphate to fall on the quiet surface of the liquid. If the end point has not been reached, a very perceptible white area is seen round the drop. This is very readily distinguished from the diminution of the slightly yellowish colour of the suspended cuprous iodide. The volume of the drop delivered by the burette must be deducted from the total volume added. After very little experience I could always obtain duplicates within 0·04 cc., the volume of the drop delivered by my burette.

The copper value of the thiosulphate is calculated as shown in the following example:

20 cc. of the copper sulphate required 27·60, 27·58 and 27·59 cc. (average, 27·59 cc.) of thiosulphate.

20 cc. of the copper sulphate $= 69{\cdot}278 \times \frac{63{\cdot}57}{249{\cdot}57} \times 20 = 352{\cdot}93$ mg. Cu.

1 cc. of thiosulphate corresponds with 12·792 mg. Cu.

This result agrees well with that obtained by the electrolytic method (average, 12·789) and that of the "nitric-talcum" process (average, 12·786). It must be noted that N/5 thiosulphate theoretically corresponds to 12·71 mg. Cu per cc. The difference is undoubtedly outside the range of experimental error. The discrepancy cannot be explained on the assumption that the thiosulphate is impure, as in several cases I found that the normality of my thiosulphate agreed very closely with theory when titrated against standard iodine prepared from potassium iodide and iodate by the addition of standard acid.

The standard heating power.

The Erlenmeyer flask in which the mixture is heated is placed on asbestos gauze over a Bunsen or Meker burner. The height of the flame and of the gauze must be such that 60 cc. of distilled water in the flask are raised from 35° to 95° in 120 ± 2 secs. Owing to variations in the gas pressure I found it necessary repeatedly to check the heating power. I tried several methods of obtaining a constant pressure, and finally adopted the manometer shown in Fig. 1. Once the apparatus is assembled an estimation can be performed without the delay of having to determine the heating power.

On an adjustable ring stand about 4 cm. above the top of the burner place an unperforated sheet of asbestos gauze. Turn on the tap *B* to its full extent. Tighten the screw *A* till the pressure is reduced about one-third. Allow the gauze to get thoroughly heated before making a test. In a 200 cc. Erlenmeyer flask of Jena glass and of about 6 cm. basal diameter place 60 cc. of distilled water. The flask is fitted with a 2-hole rubber stopper carrying a thermometer so graduated that the stem above 34° is visible above the upper edge of the stopper. The lower end of the thermometer should be about 2 mm. from the bottom of the flask. By means of a stop watch note the time for the temperature to rise from 35° to 95°. If the time is less than 120 secs. tighten the screw *A* and repeat the experiment until the desired heating power is obtained. Note the reading of the manometer. This must be observed from time to time and the screw adjusted to maintain it at the correct level as the pressure of the gas supply varies. The height of the ring and the thickness of the asbestos should be such that the pressure

is well under the minimum supplied to the laboratory and yet sufficient to prevent any risk of the flame striking back. On making a fresh set of observations within a week it is only necessary fully to open the tap *B* and to adjust the screw *A* till the previously recorded pressure is obtained. With longer intervals it is desirable to make a fresh observation of the heating power owing to the possibility of the evaporation of some of the fluid in the manometer tube.

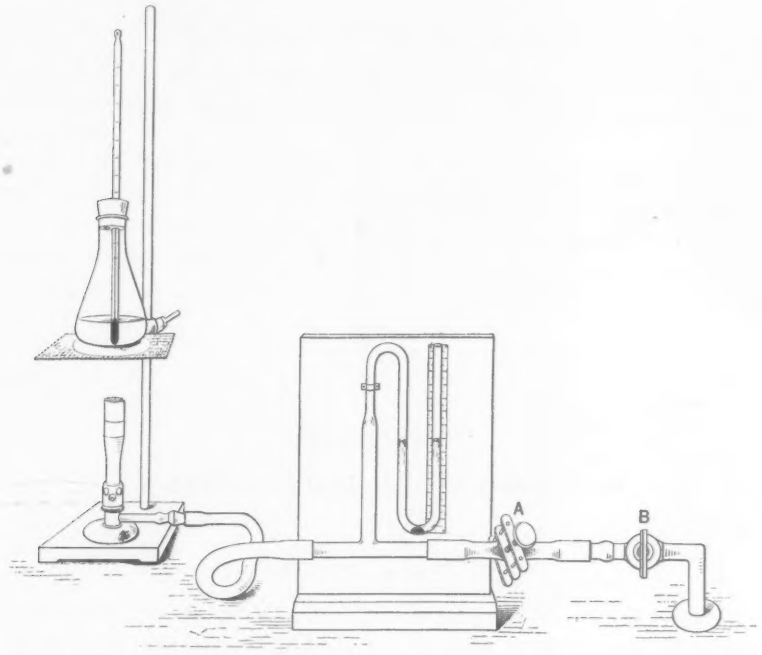

Fig. 1. Apparatus for maintaining a standard heating power. The manometer tube contains a dilute solution of eosin. It also contains a globule of mercury which nearly fills the bottom of the tube. This prevents the rapid oscillations of pressure due apparently to the explosions of local gas engines.

Filtering apparatus.

I have found it most convenient to use the apparatus shown in Fig. 2.

A is a Jena flask of 200 cc. capacity. If one is chosen with a perfectly flat bottom no trouble with fractures under pressure need be feared. The tube *B* is an ordinary calcium chloride tube. The lower end should project for

at least 3 cm. below the lower edge of the stopper to prevent loss by splashing during filtration. The filtering mat is made of glass wool, asbestos, powdered pumice and asbestos added in that order. After a test the cuprous oxide on the mat is dissolved in nitric acid diluted with an equal volume of water, and then thoroughly washed. I have used one tube for more than 100 estimations without having to add any more asbestos. The thickness of the mat should be such that the filtrate comes through under pressure in a steady stream.

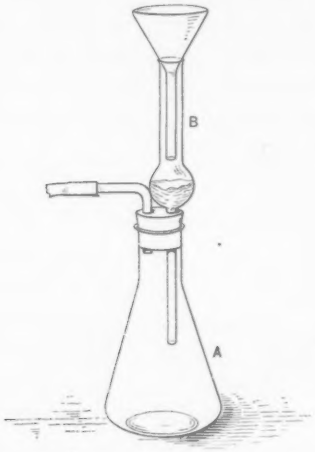

Fig. 2. Apparatus for the filtration of the reduced copper.

Method of analysis.

Into a 200 cc. Erlenmeyer flask measure 20 cc. of the standardised copper sulphate solution, 20 cc. of the alkaline tartrate and 20 cc. of the sugar solution (which must contain between 5 and 250 mg. of anhydrous lactose). Fit the two-holed rubber stopper firmly into the neck of the flask, adjust the thermometer so that its lower end is 2 mm. from the bottom of the flask and place on the heated gauze, note the time when the mercury indicates a temperature of 95°. Allow the heating to continue for exactly 20 secs. beyond this. Remove the flask by gripping the rubber stopper and swill it for a second or two under the tap or in a bowl of water. The lowering of the temperature practically stops the reduction. Filter the hot fluid at once using the stem of the thermometer as a stirring rod. Wash the flask twice with about 7 cc. of distilled water. Cool the filtrate by

holding the flask under the tap. Add exactly 4 cc. of strong sulphuric acid, insert a thermometer and cool to 20°. Add 6·5 to 7 cc. of the saturated solution of potassium iodide, washing the stem of the thermometer with this solution. Titrate at once with the standardised solution of sodium thiosulphate as described above, using soluble starch as an indicator when near the end point.

The reducing power of anhydrous lactose.

I give in table I the mean values I have obtained of the amounts of copper reduced by varying amounts of lactose. Duplicates agree to about 0·4 mg. Cu.

The stock solutions of lactose were prepared from the hydrate several times recrystallised. The values agreed exactly with those determined polarimetrically at the Cambridge University Chemical Laboratory by the kindness of Prof. Pope.

TABLE I.

Anhydrous lactose, mg.	Copper, mg.	Lactose / Copper	Anhydrous lactose, mg.	Copper, mg.	Lactose / Copper
3·9	3·3	1·182	70	88·2	0·793
5	4·4	1·136	75	94	0·798
8	7·4	1·081	80	100·9	0·793
10	9·8	1·020	85	107·3	0·792
12	13·3	0·902	90	113·6	0·792
15	16·6	0·903	95	120·1	0·791
20	24·1	0·830	100	127	0·788
25	30·5	0·820	120	152·8	0·786
30	36·9	0·813	126	160·6	0·785
35	43·2	0·810	130	166·1	0·783
40	49·9	0·802	140	179·4	0·780
45	56·1	0·802	150	191·6	0·783
50	62·5	0·800	175	223	0·785
55	69	0·805	200	255·5	0·782
57	71·3	0·800	240	307·3	0·781
60	75·2	0·798	250	320·7	0·780
65	81·3	0·799			

I have plotted these values and find that they are practically linear, except from 1 to about 20 mg. lactose. I have therefore constructed the curve shown in Fig. 3, by means of which the weight of anhydrous lactose present can be obtained from the amount of copper that it has reduced.

Example.

20 cc. of a solution of lactose were treated with 20 cc. of copper sulphate containing 348·8 mg. Cu.

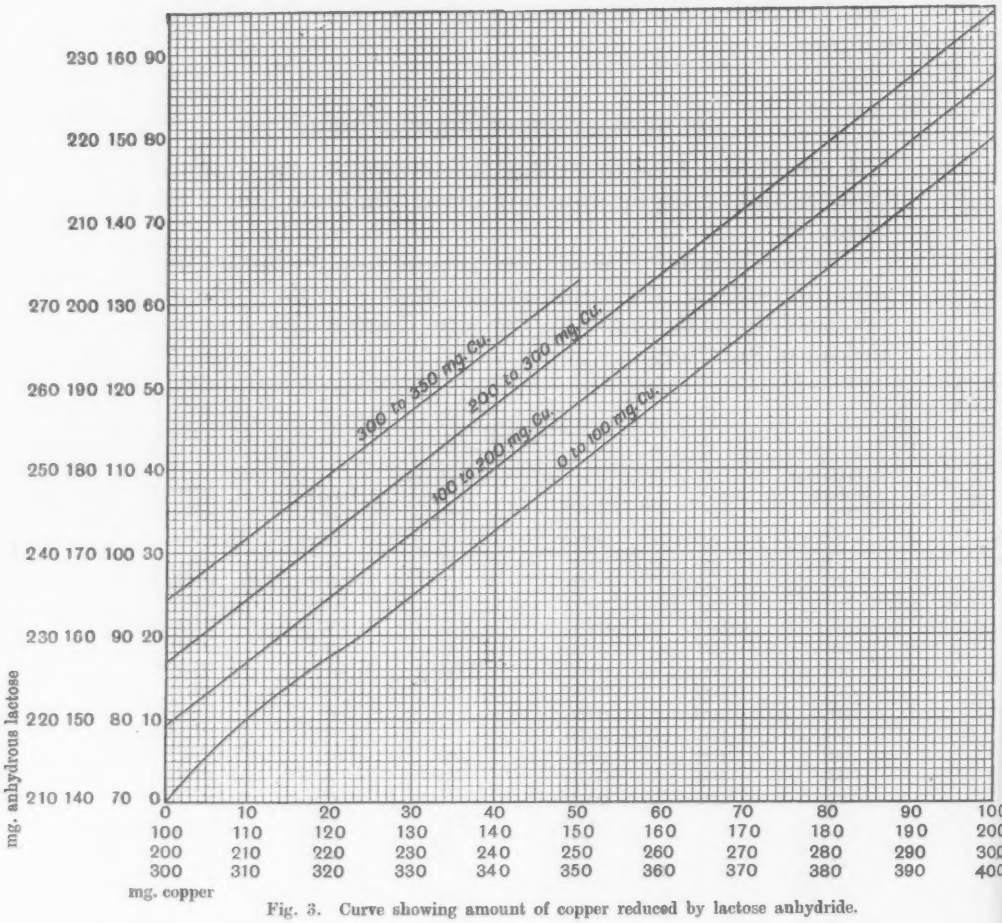

Fig. 3. Curve showing amount of copper reduced by lactose anhydride.

The copper in the filtrate liberated iodine which required 22·41 cc. of a thiosulphate, 1 cc. of which = 12·784 mg. Cu.

Cu in filtrate is $22{\cdot}41 \times 12{\cdot}784 = 286{\cdot}4$ mg.

Cu reduced is $348{\cdot}8 - 286{\cdot}4 = 62{\cdot}4$ mg.

Fig. 4. Curve showing amount of copper reduced by glucose.

On reference to the curve in Fig. 3 this is seen to correspond to 50 mg. of anhydrous lactose.

The copper values above 25 mg. Cu can be converted to anhydrous lactose by the use of the following empirical formula, deduced from the curve.

mg. anhydrous lactose = $1{\cdot}25 + 0{\cdot}778 \times$ mg. Cu.

Thus in the above example

$$\text{Lactose} = 1{\cdot}25 + 0{\cdot}778 \times 62{\cdot}4 = 49{\cdot}71 \text{ mg.}$$

This agrees fairly well with the value deduced from the curve, i.e. 50 mg.

In Fig. 4 I give the corresponding values for glucose, taking the values obtained by Peters, except that I have not given the values above 180 mg. glucose. Peters states that 200 mg. glucose reduce 349·6 mg. copper. I find that even 192 mg. glucose completely reduce the whole of the copper present in the strongest of my solutions (352·9 mg.). In the range of the curve that I give I have obtained results in very close agreement with his.

It is of some interest to note that in the case of lactose the sugar/copper ratio decreases as the amount of sugar increases. The ratio lies between 0·81 and 0·78 between 30 and 250 mg. of lactose.

In the case of glucose the ratio falls to 0·522 at 25 mg. and keeps fairly constant to about 110 mg. It then rises again.

This is not in agreement with the usual text-book statements that "the greater the excess of the copper, the greater is the amount of copper reduced by a certain amount of sugar."

The steady fall in the case of lactose seems to indicate that there is a slight hydrolysis of the sugar by the alkali, so that the amount of copper reduced by the disaccharide is relatively greater when it is present in higher concentrations. I have previously had indications of the same phenomenon when attempting to estimate lactose by the application of Benedict's method.

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XX. A VOLUMETRIC METHOD FOR THE ESTIMATION OF ETHEREAL AND INORGANIC SULPHATES IN URINE.

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Ever since the discovery by Baumann of the existence in urine of ethereal sulphates in addition to inorganic sulphates, their estimation has been carried out by weighing them as $BaSO_4$ on the principle of Baumann's original method, as modified in some more or less important details by subsequent investigators[1].

Until quite recently it has been assumed that the estimation of sulphuric acid as barium sulphate is one of the simplest and most trustworthy methods of analytical chemistry. The large amount of research which has been devoted by analytical chemists to this question [Hulett and Duschak, 1904; van 't Kruys, 1910; Allen and Johnston, 1910; Järvinen, 1913, etc.] has, however, abundantly proved that this assumption is erroneous. It has been conclusively shown that even in comparatively simple solutions the method is liable to yield inaccurate results. Without going into detail it may be stated that correct results can only be obtained by a careful adjustment of the conditions, and Allen and Johnston, indeed, attribute the many good results, which are obtainable by this method, to the happy neutralisation of inaccuracies.

If we consider that all these difficulties are met with when dealing with simple salt solutions, we are led to assume that they are still greater when we are dealing with sulphate estimations in such a complex organic fluid as urine. This was indeed recognised by O. Folin [1906] when he said: "The investigations described in this paper are the outcome of a conviction that the published records of sulphate and sulphur determinations in urine, including many of my own, are intolerably unreliable."

[1] The methods proposed by R. v. Lengyel (estimation as strontium sulphate) and by Freund (titration with barium acetate) do not seem to have acquired more than academical interest.

Folin has overcome by his careful work the difficulties inherent in the method, but there remains always the fact that, even at its best, the method is a very tedious one, requiring a great deal of time and experience if accurate results are desired.

We have worked out a volumetric method for the estimation of ethereal and inorganic sulphates in urine, which is based on the well-known insolubility of the sulphate of the base benzidine (*p*-diamino-diphenyl $NH_2.C_6H_4.C_6H_4.NH_2$). The method seems to be free from the objections of the barium method and has the advantage of being very rapid, whilst the results are at least as trustworthy as those obtained by the Baumann-Folin method, which we have adopted as a standard.

The principle of the method depends on the fact that insoluble benzidine sulphate is precipitated from solutions of sulphates on the addition of a soluble benzidine salt. As benzidine is a weak base, its salts with strong acids are readily dissociated and the sulphuric acid contained in benzidine sulphate may be quantitatively titrated with standard alkali solutions, using phenolphthalein as an indicator. The method was introduced in this form into inorganic analysis by Raschig [1903] and its reliability was confirmed by v. Knorre [1905, 1910] and by Friedheim and Nydegger [1907]. Järvinen [1913] came to the conclusion that the benzidine method gives the most trustworthy results and is preferable to the old barium method in inorganic analysis.

Before the benzidine method can be applied to urine it is necessary to investigate if it fulfils the essential conditions which are demanded. Any reagent which is intended to replace barium chloride for the estimation of ethereal and inorganic sulphates in urine must fulfil three conditions. (1) It must precipitate in the cold only the inorganic sulphates, free from ethereal sulphates. (2) It must not precipitate any other urinary constituents. (3) The precipitation of the total sulphates after hydrolysis (= inorganic + ethereal sulphates) must be complete.

With regard to the first condition we have tested the behaviour of benzidine solutions towards several ethereal sulphates which we prepared synthetically according to Baumann [1878]. It was found that aqueous solutions of the potassium salts of phenylsulphuric acid, *p*-cresylsulphuric acid and resorcinolsulphuric acid behave towards benzidine hydrochloride exactly in the same way as towards barium chloride, i.e. the organically bound sulphuric acid is not precipitated in the cold, but readily after boiling for a short time with dilute hydrochloric acid. We have further tested the behaviour of the ethereal sulphates contained in normal urine towards

benzidine solutions in the following way. 200 cc. of urine were freed from inorganic sulphates by precipitation with barium chloride in the usual way. The filtrate which contained only the ethereal sulphates remained perfectly clear after the addition of a solution of benzidine hydrochloride. We must therefore conclude that under the ordinary conditions of urinary analysis the ethereal sulphates are not precipitated by benzidine.

With regard to the second condition we have found that the addition of a benzidine solution to normal urine results at once in the production of a bulky cream coloured precipitate, which on examination is seen to be partially crystalline and partially amorphous. It can easily be shown that this precipitate contains benzidine phosphate besides benzidine sulphate. This fact alone would seem *a priori* to condemn the method. (See also K. Spiro [Neubauer-Huppert, 1910], which is the only reference to the subject we came across in the literature.) We found, however, that urine which is faintly acidified with dilute hydrochloric acid, gives a crystalline precipitate which rapidly settles. This precipitate consists entirely of crystals of benzidine sulphate. It was tested qualitatively with negative results for phosphoric and uric acids, and the absence of other acids than sulphuric acid was proved by the close agreement of control analyses in which sulphuric acid determinations were carried out in the same sample by the barium method and by direct titration. The experiment described above, in which it was shown that the addition of benzidine solution to urine freed from inorganic sulphates produces no further precipitate, may also be quoted as evidence that sulphates are the only urinary constituents which are precipitated from acid urine by benzidine.

With regard to the completeness of the precipitation of sulphuric acid by benzidine in the presence of the other constituents of urine, we consider this proved by experiments in which added sulphuric acid was quantitatively recovered, and further by the fact that after the removal of the benzidine precipitate no further precipitate was produced by the addition of barium chloride.

Benzidine seems therefore to fulfil the conditions laid down above and we felt justified in proceeding to the actual quantitative estimations. It now became necessary to carry out a series of experiments in order to investigate (1) the influence of increasing quantities of hydrochloric acid on the accuracy of the result, (2) the minimum time necessary for complete precipitation, and (3) the quantity of benzidine solution required for complete precipitation.

The results of these experiments showed that excess of hydrochloric acid prevents the precipitation of benzidine sulphate and that a quantity sufficient

to produce an acid reaction to Congo red paper gives accurate results. Further it was found that the precipitation is complete after five minutes and that one hour's standing does not influence the result. Even a large excess of benzidine solution does not affect the accuracy of the result.

The ethereal and inorganic sulphates in urine may obviously be estimated in several ways by means of benzidine. (1) Inorganic and total sulphates may be estimated directly and the amount of ethereal sulphates be calculated by the difference between the two. (2) Total and ethereal sulphates may be estimated directly and inorganic sulphates calculated by difference. (3) Inorganic and ethereal sulphates may be estimated separately.

As the direct estimation of ethereal sulphates, owing to the small amount present, requires a relatively large volume of urine, which is not always available, we have estimated them indirectly by the difference between the total and inorganic sulphates, a procedure which is analogous to that employed by Folin in the barium chloride method.

In our experience the practical application of the benzidine method to urine does not meet with any difficulties if the precautions are observed, which have already been described by Raschig and by Järvinen in the case of inorganic analysis. They refer to care in filtration and washing of the precipitate. It is also important that an excess of free hydrochloric acid be avoided. Partly for this reason and partly in order to avoid the excessive formation of dark pigments which might interfere with the end point of titration, we have found it advisable to reduce the amount of hydrochloric acid in the hydrolysis of the ethereal sulphates to the minimum necessary for complete hydrolysis.

It is evident that the benzidine method can also be employed for the estimation of the neutral sulphur, by subtracting the quantity of total sulphates from that of the total sulphur. The latter may be estimated by applying the benzidine method to the final solution of mineral salts, as obtained by Wolf and Oesterberg's modification [1910] of Benedict's method [1909]. The presence of copper in this solution does not in any way interfere with the accuracy of the results.

By working with 0·01 N KOH solutions and methyl-red as an indicator it seems also possible to use much smaller quantities of urine and to convert the method into a micro-analytical method. Such a method would be of use in metabolic experiments on small animals and we are still engaged with experiments in this direction.

Experimental.

The preparation of the benzidine solution. 4 g. benzidine (Kahlbaum) are rubbed into a fine paste with about 10 cc. of water and transferred with about 500 cc. of water into a 2 litre flask. 5 cc. of concentrated HCl (spec. grav. 1·19) are added and the solution made up to 2 litres with distilled water. 150 cc. of this solution, which keeps indefinitely, are sufficient to precipitate 0·1 g. H_2SO_4.

Influence of acidity, time and volume. The following series of experiments was carried out on a large sample of mixed urine. 25 cc. of urine were taken in each case. An estimation of inorganic sulphates by the barium method gave 0·104 % SO_3.

Influence of increasing quantities of HCl (1 : 4).

HCl, cc.	Benzidine solution, cc.	Time of standing	0·1 N KOH, cc.	SO_3 %
1	100	10 mins.	6·45	0·103
2	,,	,,	6·50	0·104
3	,,	,,	6·30	0·101
4	,,	,,	6·00	0·096
5	,,	,,	5·60	0·089
7	,,	,,	4·60	0·074
10	,,	,,	2·20	0·035
15	,,	,,	none	—

This table shows clearly that an excess of hydrochloric acid prevents the formation of the benzidine sulphate precipitate. The quantity of hydrochloric acid necessary to produce an acid reaction with Congo red paper was 0·8 cc. In all the subsequent experiments we used therefore 1–2 cc. HCl (1 : 4).

Influence of time of standing.

HCl, cc.	Benzidine solution, cc.	Time of standing	0·1 N KOH, cc.	SO_3 %
2	100	5 mins.	6·40	0·102
,,	,,	10 ,,	6·45	0·103
,,	,,	15 ,,	6·40	0·102
,,	,,	20 ,,	6·60	0·107
,,	,,	30 ,,	6·45	0·103
,,	,,	45 ,,	6·45	0·103
,,	,,	60 ,,	6·50	0·104

These results prove that precipitation is complete after five minutes and that the result is not affected by one hour's standing. The short time

required for precipitation is a distinct advantage as against the lengthy period usually required before barium sulphate precipitates can be filtered.

Influence of volume of benzidine solution.

HCl, cc.	Benzidine solution, cc.	Time of standing	0·1 N KOH, cc.	SO_3 %
2	70	5 mins.	6·50	0·104
,,	100	,,	6·40	0·102
,,	150	,,	6·50	0·104
,,	180	,,	6·50	0·104
,,	200	,,	6·50	0·104

The final result is evidently not influenced by an excessive quantity of the reagent and 100 cc. of the standard benzidine solution are sufficient to precipitate the whole of the sulphates contained in 25 cc. of normal urine.

An experiment may be mentioned here in which a known quantity of sulphate was purposely added to the urine. 2 cc. of a dilute sulphuric acid required 10·9 cc. 0·1 N KOH for neutralisation. This solution of sulphate, containing 53·4 mg. H_2SO_4, was added to 25 cc. urine which was previously found to contain 31·9 mg. H_2SO_4. In two experiments the final benzidine precipitates required 17·4 and 17·5 cc. 0·1 N KOH = 85·3 mg. and 85·4 mg. H_2SO_4.

	H_2SO_4 added	H_2SO_4 found
(1)	53·4 mg.	53·4 mg.
(2)	53·4 mg.	53·5 mg.

This experiment not only proves that the organic constituents of the urine do not interfere with the precipitation of sulphates by benzidine, but also that the precipitation is quantitative.

As an outcome of these preliminary experiments we have finally adopted the following procedure:

1. *The estimation of inorganic sulphates.*

25 cc. of urine are measured into a 250 cc. Erlenmeyer flask and acidified with dilute hydrochloric acid (1 : 4) until the reaction is distinctly acid to Congo red paper. Usually 1–2 cc. of dilute acid were added. 100 cc. of the benzidine solution (see above) are then run in and the precipitate, which forms in a few seconds, allowed to settle for ten minutes. The precipitate is filtered under pressure and washed with 10–20 cc. of water, saturated with benzidine sulphate. The precipitate and filter paper are transferred into the original precipitation flask with about 50 cc. water and titrated hot with

0·1 N KOH, after the addition of a few drops of a saturated alcoholic solution of phenolphthalein. 1 cc. 0·1 N KOH corresponds to 49 mg. H_2SO_4.

2. *The estimation of total sulphates (inorganic and ethereal).*

The hydrolysis of the ethereal sulphates may be carried out in the usual way by gently boiling 25 cc. of urine with 20 cc. dilute HCl (1 : 4) for fifteen to twenty minutes. In this case it is necessary carefully to neutralise the solution after boiling and to add again dilute HCl until the reaction is acid to Congo red. We obtained, however, equally good results by reducing the quantity of acid used for hydrolysis to 2–2·5 cc. HCl (1 : 4), adding also 20 cc. of water to prevent undue concentration. The hydrolysis is complete after 15–20 minutes' gentle boiling and the precipitation of the cold solution may be proceeded with at once without previous neutralisation. It seems also that the smaller quantity of acid produces only very little pigment, which interferes to a slight extent with the end point, if the larger amount of acid is used.

The filtration and titration of the precipitate is carried out exactly as under 1.

In order to obtain accurate results, it is most important that the precipitate should be finely suspended in water before titration, and this again entails certain precautions during filtration, so as to prevent the caking together of the precipitate. We used a funnel of 6 cm. diameter and a perforated porcelain filter plate (5–7 mm.), covered either with paper pulp or with a well-fitting filter paper. Filtration and washing are carried out under pressure with the precaution of not allowing the precipitate at any time to be sucked dry on the filter. When the filtrate is no longer acid to Congo red and the precipitate together with the filter paper have been transferred to the original flask, it is necessary to shake it thoroughly before titration. Raschig recommended closing the flask with a rubber stopper for this purpose, a precaution which we found not essential. It is necessary to employ a stronger phenolphthalein solution than usual, as it is adsorbed to a considerable extent by the filter paper.

We have tested the accuracy of the method by comparing the results of the benzidine method with those obtained by Folin's gravimetric barium method. As many as seventy-five separate analyses were carried out. Most of the results agreed absolutely, the largest difference amounted to − 0·0025 % in favour of the barium method and to + 0·001 % in favour of the benzidine method (calculated as SO_3 % of urine).

Some of the results, taken at random, may be quoted in full. 25 cc. of urine were used in each case.

	Gravimetric		Volumetric	
	Mg. $BaSO_4$	% SO_3	Cc. 0·1 N KOH	% SO_3
(*a*) *Inorganic sulphates.*				
Urine 1	87·1	0·120	7·54	0·120
	86·7	0·119	(6 identical	
		Mean 0·1195	estimations)	
Urine 2	57·6	0·079	4·90	0·078
	58·7	0·081	5·00	0·080
		0·080		0·079
Urine 3	85·7	0·117	7·28	0·116
			7·36	0·118
			7·32	0·117
			7·32	0·117
				0·117
(*b*) *Total sulphates.*				
Urine 3	93·1	0·128	7·99	0·128
			8·08	0·129
			8·08	0·129
				0·129

It will be seen that the results of the benzidine method agree not only *inter se* but also with those of the barium method as closely as can be expected[1].

Summary.

A rapid and accurate method for the estimation of sulphates in urine consists in their precipitation with benzidine solution and the subsequent titration of the insoluble benzidine sulphate by means of 0·1 N potassium hydrate.

[1] A full account of this method was given at the November meeting of the Biochemical Society at King's College, London. Since this paper was ready for the press, our attention has been drawn to a publication on the same subject by R. Gauvin and V. Skarzynski in the December number of the *Bull. Soc. Chim.* p. 1121. It is satisfactory to note that these authors arrived at practically the identical method and results, although they do not seem to have systematically investigated the requisite conditions.

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XXI. A NOTE ON THE PRODUCTION OF CASEIN FROM CASEINOGEN.

By SAMUEL BARNETT SCHRYVER.

(*Received March 7th, 1914.*)

In the last number of this Journal a paper by A. Harden and A. B. Macallum [1914] appeared, in which certain experiments on the production of casein from caseinogen are described. They draw the conclusion that this process is accompanied by a cleavage of nitrogen, phosphorus and calcium from the latter substance. These results are not in accord with my own [1913, 1, 2] and those independently obtained by Bosworth [1913]. I should like to draw attention to the reason of this apparent discrepancy. My own results were obtained with a solution of caseinogen in half-saturated lime-water, which had been obtained by rotating the lime-water with excess of caseinogen for several hours, and was consequently saturated with the acid and had a strongly acid reaction. Harden and Macallum, on the other hand, ground up their caseinogen preparation with excess of calcium carbonate and obtained thereby a solution of the basic caseinogenate. Now the acid solutions obtained by my method give clots with extraordinary readiness without addition of a soluble calcium salt when treated with rennin, even at ordinary room temperature. They also give clots with calcium chloride alone within certain limits of concentration when very gently warmed (to 25°). A condition for this very rapid clot formation is that the solution should be saturated with caseinogen. The rapidity of clot formation diminishes as soon as the acidity of the solutions is neutralised with calcium hydroxide. Under the conditions under which I worked, clots were obtained, the free acids from which had precisely the same empirical composition (N and P content and acidity) as caseinogen. The products were, however, only half as soluble in lime-water as caseinogen, and the solutions thus obtained could no longer be got to clot on rennin addition. A large number of attempts to produce a clottable solution from casein have been made, but without success. On the

question of the irreversibility of the casein formation, I am in accord with Harden and Macallum. I still think it quite likely that the casein is a compound of caseinogen with the ferment, although this suggestion has not yet been definitely proved.

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XXII. HERZIG AND MEYER'S REACTION APPLIED TO PROTEINS AND AMINO-ACIDS.

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(*Received March 9th, 1914.*)

This reaction [Herzig and Meyer, 1894] has been known for a considerable time as a means of detecting the presence of, and estimating, methyl groups attached to nitrogen in organic compounds. It consists in heating the substance to a high temperature in the presence of excess of hydriodic acid, so that the methyl groups leave the nitrogen to form methyl iodide, and the hydrogen of the hydriodic acid takes the place of the methyl group. The methyl iodide is then carried over into alcoholic silver nitrate, where silver iodide is formed. The precipitate of silver iodide can be weighed and from its amount the percentage of methyl attached to nitrogen can be calculated.

Certain precautions have to be taken in the estimation of methylated nitrogen in this way. Thus substances containing methoxy groups give a silver iodide precipitate, according to the well known Zeisel reaction. The precipitate from methoxy groups, however, forms when the reaction mixture is heated to 130°, whereas that from methylamino groups requires a temperature of 230°. In connexion with methoxy derivatives it must also be remembered that glycerol and fats (on account of their glycerol content) give rise to isopropyl iodide and to a silver iodide precipitate. Trier has published a criticism [Trier, 1913] of the method stating that amino-ethyl alcohol gives a precipitate under these conditions.

In the course of ordinary estimations by this method, it was found that commercial gelatin gave a positive result. At the end of the experiment the precipitate formed in the flask containing silver nitrate was black. This was due to the sulphur in the protein, for on boiling with dilute nitric acid, the black colour disappeared, leaving a clean precipitate of silver iodide. It was similarly found that Hammarsten's "Casein" gave the reaction, after

having been first carefully extracted with ether to make sure of the absence of fat. These results seemed to point to the possibility of the occurrence in protein of methylated nitrogen. The reaction could not be due to methoxy derivatives, as the precipitate of silver iodide was only formed when the reaction mixture was heated to about 230°.

To investigate this further, a dried pancreatic digest of protein, also carefully extracted with ether, was examined. This too gave a positive result, as did also a phosphotungstic precipitate from the digest, and the filtrate from the precipitation. It seemed desirable at this stage to make sure of the soundness of the reaction when applied to individual known amino-acids. Instead of the negative results expected, it was found that a mixture of glycine, tryptophane and a leucine-valine fraction gave a silver iodide precipitate. Of these separately, glycine and the leucine-valine fraction were positive, whilst tryptophane was negative.

On the basis that 100 grams of silver iodide correspond to 6·38 grams of methyl, the following percentages of methyl (CH_3) present in combination with nitrogen were found:

Glycine	0·34 %
	0·46
Alanine	0·43
	0·63
Leucine-valine	0·68
	0·95
Histidine	0·03
Tyrosine	negative
Tryptophane	negative
Commercial gelatin ...	0·77 %
Hammarsten's casein ...	0·97

In the case of the amino-acids the explanation seems to be that after the hydriodic acid has been distilled away, during the dry heating at 250°–270°, simple amino-acids break up, giving off carbon dioxide and leaving an amine, which should quite properly give the reaction. Thus glycine would decompose as follows:

$$NH_2 . CH_2 . COOH = NH_2 . CH_3 + CO_2.$$

Such an explanation would seem to apply even to leucine. If it broke up in this way, isoamylamine would be formed; and in fact when isoamylamine was tested by the reaction, a silver iodide precipitate was formed in amount corresponding to the above percentages.

It will be noticed that the values for protein are slightly greater than those for the individual amino-acids, and yet it is apparently only the amino-acids of simple constitution which do give a positive result. It may therefore

be argued that as these can only form a small fraction of the protein, the high values with protein are only partly explained. It is indeed possible that methylamino groupings do exist in protein, but this reaction does not afford a sufficiently reliable test for their presence. Quantitatively the limits of error are as wide as +3% and −15%. Thus sarcosine, which on the above considerations might be expected to give a higher value than the theoretical 16·8% for methyl attached to nitrogen, actually gave 14·4%. When it is seen that simple amino-acid groups are broken up at the high temperature of 250° and in the presence of hydriodic acid, the likelihood that there are other groupings in protein itself (not methylamino) which will also break up becomes extremely great.

The work out of which this note has arisen was begun during the tenure of the Michael Foster Studentship in Cambridge University. My thanks are due to Dr F. G. Hopkins for suggesting the work and for supervising its earlier stages.

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XXIII. STUDIES IN PROTEIN HYDROLYSIS.

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(*Received March 11th, 1914.*)

It is well known that proteins are built up of various proportions of different amino-acids, and that on hydrolysis they are broken down, giving proteoses, peptones, polypeptides, and finally their constituent amino-acids. The study of the ultimate products of hydrolysis has been extensively pursued, but, as yet, little light has been thrown upon the initial stages of the hydrolytic decomposition, and it was with the view of finding out something about the earlier steps in the degradation of the protein molecule, and incidentally to compare the behaviour of two proteins under the same conditions, that the following work was carried out.

The two proteins used were caseinogen and egg albumin; they were hydrolysed by strong hydrochloric acid for short intervals of time, and the various amounts of ammonia, amino-nitrogen, and nitrogen in bodies precipitated from the resulting liquid by phosphotungstic acid, were determined after each hydrolysis, the values so obtained being plotted on a curve.

The caseinogen used was "Merck's Pure" which had been extracted for several days with boiling alcohol in order to remove all traces of substances soluble in alcohol. It contained 14·92 per cent. of nitrogen and had an ash content of 4·27 per cent. so that the percentage of nitrogen in the ash free substance was 15·59.

The egg albumin used was prepared in the pure crystalline form from the whites of fresh eggs, Hopkins' modification of Hofmeister's method being used for the preparation. The crystalline product was twice purified by solution and precipitation, coagulated by absolute alcohol, washed free from ammonium sulphate, and carefully dried at 110° in an air oven. It contained 15·36 per cent. of nitrogen as determined by Dumas' method (Kjeldahl's method gave 14·15 per cent. nitrogen) and was practically ash free, containing 0·005 per cent. ash.

Method of experiment.

About one gram of protein was carefully weighed out into a hard glass, round-bottomed flask, fitted with a cork and glass tube to act as an air condenser, 20 cc. of boiling strong hydrochloric acid were added and the flask placed on a heated sand bath. When the time of hydrolysis (taken from the moment of adding the boiling hydrochloric acid) was completed, the flask was removed from the sand bath and at once cooled by the addition of cold water.

Estimation of ammonia (amide nitrogen).

The liquid was then introduced into a steam distillation flask, containing excess of a paste of magnesium oxide, the ammonia distilled off in steam into excess of standard acid, and the excess of acid titrated with standard alkali, using methyl orange as indicator.

This method of estimating the amide nitrogen of proteins, first used by Hausmann [1899 and 1900] was employed because, although it is well known that cystine is somewhat easily decomposed at 100°, yet it has been shown by Osborne that caseinogen and egg albumin, proteins which contain only minute quantities of cystine, if any, give the same results for amide nitrogen, whether the ammonia is distilled off at 100° by magnesia or *in vacuo* at low temperature by lime.

Humin nitrogen.

The liquid remaining in the flask was then filtered off from the magnesia, which was carefully washed with hot water until the washings were free from chloride. The filtrate and washings were then evaporated down and made up to 250 cc.

The magnesia precipitate was then dried, carefully detached from the filter paper and the nitrogen in it determined by Kjeldahl's method.

Amino-nitrogen (NH_2 *groups*).

This was estimated by Sörensen's [1908] formaldehyde method. To determine the amount of amino-nitrogen, a definite quantity (25 cc.) of the filtrate from the magnesia was made just faintly alkaline to phenolphthalein by adding dilute N/20 hydrochloric acid to the alkaline liquid. Then to this faintly alkaline solution were added 5 cc. of formalin (proved to be in

excess of what was actually required) made alkaline to the same faint pink colour by adding N/20 soda. The pink colour disappeared at once on mixing the solutions and the acidity produced was exactly measured by titrating with N/50 soda, the end point being taken when the solution reached the same tint as had been used previously.

The solutions were only slightly yellow in colour (due to humin substances in solution) and in no case was the colour sufficiently deep to necessitate clarifying the liquid, before titrating to the pink colour.

The titrations agreed very well among themselves, despite the very dilute alkali used, which tended to make the end point of the titration somewhat indefinite.

Diamino and polypeptide nitrogen, i.e. nitrogen precipitated by phosphotungstic acid in sulphuric acid solution.

This was estimated by precipitating in acid solution with phosphotungstic acid dissolved in dilute sulphuric acid, and then carrying out a Kjeldahl estimation on the carefully washed precipitate. 75 cc. of the liquid were used, made acid with dilute sulphuric acid, and excess of a 25 per cent. solution of phosphotungstic acid in 5 per cent. sulphuric acid added. The beakers containing the mixture were allowed to stand for 24 hours, during which time the precipitate had settled to the bottom leaving a clear liquid above. This was decanted and the precipitate washed with 5 per cent. phosphotungstic acid solution in 5 per cent. sulphuric acid, allowed to stand till the precipitate had again settled to the bottom of the beaker, and the clear liquid again decanted. Finally the precipitate was washed on to a filter, dried, and the nitrogen in it estimated by Kjeldahl's method, deduction for the nitrogen contained in the filter paper being made in each case.

It was found that the estimation of the nitrogen precipitated by phosphotungstic acid was a very difficult matter and for some time the duplicates did not agree. However, when the procedure above was adhered to, the results obtained agreed moderately well. The two chief difficulties to be overcome were the matter of washing all the precipitates in exactly the same way and for the same length of time, and then getting them filtered properly. The filtering was a very tedious business for, if great care was not exercised, the precipitate came through a Buchner filter, while it filtered extremely slowly through an ordinary filter paper. Finally, filtering through a Buchner funnel was adopted, great care being exercised not to get too big a pressure on the filter pump.

Experimental results.

I. *Estimation of Ammonia.* A. *Caseinogen.*

Time of hydrolysis in hours	Weight of caseinogen, g.	Number of cc. 0·1N HCl	Equivalent nitrogen, g.	Percentage nitrogen	Mean of duplicates
0	0·8376	0·20	0·00028	0·033	0·042
	0·9678	0·35	0·00049	0·051	
0·25	0·9994	9·55	0·01337	1·340	1·340
	1·0613	10·20	0·01428	1·340	
0·50	1·1179	10·90	0·01526	1·360	1·360
	0·9648	9·35	0·01309	1·360	
1	1·0627	10·60	0·01484	1·390	1·385
	1·0769	10·60	0·01484	1·380	
2	0·9842	9·90	0·01386	1·410	1·405
	1·1830	11·80	0·01652	1·400	
3	0·9467	9·65	0·01351	1·430	1·440
	0·9553	9·90	0·01386	1·450	
4	0·9026	9·40	0·01316	1·460	1·460
	0·9896	10·35	0·01449	1·460	
5	0·9373	10·10	0·01414	1·510	1·505
	1·0038	10·85	0·01519	1·500	
6	1·2733	13·50	0·01890	1·490	1·495
	1·0307	11·10	0·01554	1·500	

I. *Estimation of Ammonia.* B. *Egg albumin.*

Time of hydrolysis in hours	Weight of egg albumin, g.	Number of cc. 0·1N HCl used	Equivalent nitrogen, g.	Percentage nitrogen	Mean of duplicates
0	0·8824	0·40	0·00056	0·063	0·063
0·25	0·9942	6·70	0·00938	0·940	0·940
	1·0085	6·80	0·00952	0·940	
0·50	0·8607	6·10	0·00854	0·990	0·975
	0·9895	6·80	0·00952	0·960	
1	0·8164	6·20	0·00868	1·060	1·050
	1·0275	7·65	0·01710	1·040	
2	0·8806	6·90	0·00966	1·100	1·100
	0·7636	6·05	0·00847	1·100	
3	1·0956	9·10	0·01274	1·160	1·150
	0·9860	8·00	0·01120	1·140	
4	0·7748	6·70	0·00938	1·210	1·200
	1·0865	9·20	0·01288	1·190	
5	0·9522	8·45	0·01183	1·250	1·245
	1·0181	9·00	0·01260	1·240	
6	1·0429	9·20	0·01288	1·240	1·260
	1·0098	9·10	0·01274	1·280	
7	0·9884	8·90	0·01246	1·260	1·260
	0·8228	7·40	0·01036	1·260	

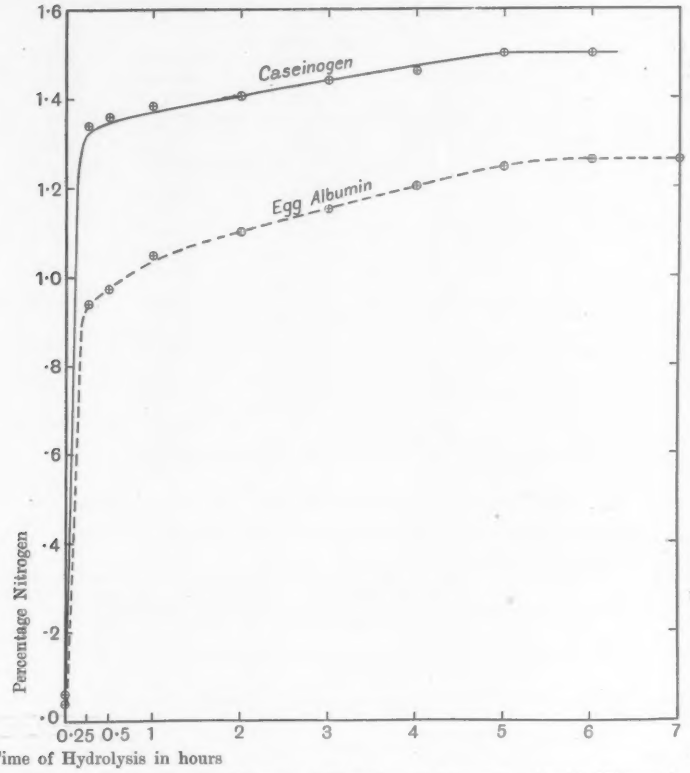

Fig. 1. Curves showing the rate of liberation of Ammonia. (Amide Nitrogen.)

II. *Estimation of Humin Nitrogen.* A. *Caseinogen.*

Time of hydrolysis in hours	Weight of caseinogen, g.	Number of cc. 0·1 N HCl used	Equivalent nitrogen, g.	Percentage nitrogen	Mean of duplicates
0·25	0·9994	4·75	0·00665	0·67	0·625
	1·0613	4·40	0·00616	0·58	
0·50	1·1179	2·60	0·00364	0·33	0·320
	0·9648	2·10	0·00294	0·31	
1	1·0627	2·50	0·00350	0·33	0·340
	1·0769	2·70	0·00378	0·35	
2	0·9842	1·60	0·00224	0·23	0·235
	1·1830	2·00	0·00280	0·24	
3	0·9467	1·90	0·00266	0·28	0·290
	0·9553	2·00	0·00280	0·30	
4	0·9026	1·80	0·00252	0·28	0·280
	0·9896	2·00	0·00280	0·28	
5	0·9373	1·50	0·00210	0·22	0·240
	1·0038	1·90	0·00264	0·26	
6	1·2733	2·90	0·00406	0·32	0·310
	1·0307	2·20	0·00308	0·30	

Mean of values (excluding 0·25 hour) = 0·29 per cent.

II. *Estimation of Humin Nitrogen.* B. *Egg albumin.*

Time of hydrolysis in hours	Weight of egg albumin, g.	Number of cc. 0·1 N HCl used	Equivalent nitrogen, g.	Percentage nitrogen	Mean of duplicates
0·25	0·9942	4·30	0·00602	0·61	
	1·0085	11·90	0·01666	1·65	
0·50	0·8607	2·00	0·00280	0·32	0·31
	0·9895	2·10	0·00294	0·30	
1	0·8164	2·10	0·00294	0·36	0·32
	1·0576	2·10	0·00294	0·28	
2	0·8806	1·90	0·00266	0·30	0·30
	0·7636	1·60	0·00224	0·30	
3	1·0956	2·15	0·00301	0·28	0·28
	0·9860	2·00	0·00280	0·28	
4	0·7748	1·90	0·00266	0·34	0·33
	1·0865	2·50	0·00350	0·32	
5	1·0181	3·00	0·00420	0·41	0·36
	0·9522	2·15	0·00301	0·31	
6	1·0098	1·95	0·00273	0·27	0·27
	1·0429	2·00	0·00280	0·27	
7	0·9884	2·00	0·00280	0·28	0·32
	0·8228	2·10	0·00294	0·36	

Mean of values (excluding 0·25 hour) = 0·31 per cent. nitrogen.

III. *Estimation of Amino-nitrogen.* A. *Caseinogen.*

25 cc. of the standard volume of liquid were titrated in each case with N/50 soda.

Time of hydrolysis in hours	Weight of caseinogen, g.	Number of cc. N/50 soda used	Equivalent nitrogen, g.	Percentage nitrogen	Mean of duplicates
0·25	0·9994	$\left.\begin{matrix}11·30\\11·10\end{matrix}\right\} \times 10 = 112·00$	0·03136	3·14	3·190
	1·0613	$\left.\begin{matrix}12·30\\12·25\end{matrix}\right\} \times 10 = 122·75$	0·03447	3·24	
0·50	1·1179	$\left.\begin{matrix}14·65\\14·75\end{matrix}\right\} \times 10 = 147·00$	0·04116	3·68	3·705
	0·9648	$\left.\begin{matrix}12·85\\12·85\end{matrix}\right\} \times 10 = 128·50$	0·03598	3·73	
1	1·0627	$\left.\begin{matrix}18·45\\18·45\end{matrix}\right\} \times 10 = 184·50$	0·05166	4·86	4·885
	1·0769	$\left.\begin{matrix}18·95\\18·85\end{matrix}\right\} \times 10 = 189·00$	0·05292	4·91	
2	0·9842	$\left.\begin{matrix}19·30\\19·40\end{matrix}\right\} \times 10 = 193·50$	0·05418	5·50	5·525
	1·1830	$\left.\begin{matrix}23·35\\23·55\end{matrix}\right\} \times 10 = 234·50$	0·06566	5·55	
3	0·9467	$\left.\begin{matrix}20·20\\20·30\end{matrix}\right\} \times 10 = 202·50$	0·05670	6·00	6·020
	0·9553	$\left.\begin{matrix}20·60\\20·60\end{matrix}\right\} \times 10 = 206·00$	0·05768	6·04	
4	0·9026	$\left.\begin{matrix}20·50\\20·80\end{matrix}\right\} \times 10 = 206·50$	0·05782	6·40	6·350
	0·9896	$\left.\begin{matrix}22·25\\22·25\end{matrix}\right\} \times 10 = 222·50$	0·06230	6·30	
5	0·9373	$\left.\begin{matrix}21·75\\21·85\end{matrix}\right\} \times 10 = 218·00$	0·06104	6·51	6·565
	1·0038	$\left.\begin{matrix}23·45\\23·30\end{matrix}\right\} \times 10 = 233·75$	0·06650	6·62	
6	1·2733	$\left.\begin{matrix}24·90\\24·70\end{matrix}\right\} \times 10 = 248·00$	0·08694	6·74	6·785
	1·0307	$\left.\begin{matrix}31·10\\31·00\end{matrix}\right\} \times 10 = 310·50$	0·06944	6·83	

III. *Estimation of Amino-nitrogen.* B. *Egg albumin.*

25 cc. of the standard volume (250 cc.) of liquid were titrated in each case with N/50 soda.

Time of hydrolysis in hours	Weight of egg albumin used, g.	Number of cc. of N/50 soda used	Equivalent nitrogen, g.	Percentage nitrogen	Mean of duplicates
0·25	0·9942	5·00 / 4·80 × 10 = 49·00	0·01372	1·38	1·400
	1·0085	5·15 / 5·05 × 10 = 51·00	0·01428	1·42	
0·50	0·8607	9·60 / 9·50 × 10 = 95·50	0·02674	3·09	3·045
	0·9895	10·30 / 10·50 × 10 = 104·00	0·02912	3·00	
1	0·8164	14·40 / 14·30 × 10 = 143·50	0·04018	4·93	4·930
	1·0576	18·20 / 18·30 × 10 = 182·50	0·05210	4·93	
2	0·8806	17·20 / 17·30 × 10 = 172·50	0·04816	5·47	5·540
	0·7636	15·40 / 15·20 × 10 = 153·00	0·04284	5·61	
3	1·0956	22·90 / 23·10 × 10 = 230·00	0·06440	5·88	5·900
	0·9860	20·90 / 20·80 × 10 = 208·50	0·05838	5·92	
4	0·7748	16·40 / 16·60 × 10 = 165·00	0·04620	6·00	6·100
	1·0865	24·10 / 24·05 × 10 = 240·75	0·06741	6·20	
5	1·0181	22·90 / 23·30 × 10 = 231·00	0·06496	6·38	6·430
	0·9522	22·00 / 22·10 × 10 = 220·50	0·06174	6·48	
6	1·0098	23·80 / 23·85 × 10 = 238·25	0·06671	6·66	6·605
	1·0429	24·30 / 24·50 × 10 = 244·00	0·06832	6·55	
7	0·9884	23·70 / 23·80 × 10 = 237·50	0·06650	6·74	6·765
	0·8228	20·00 / 19·80 × 10 = 199·00	0·05572	6·77	

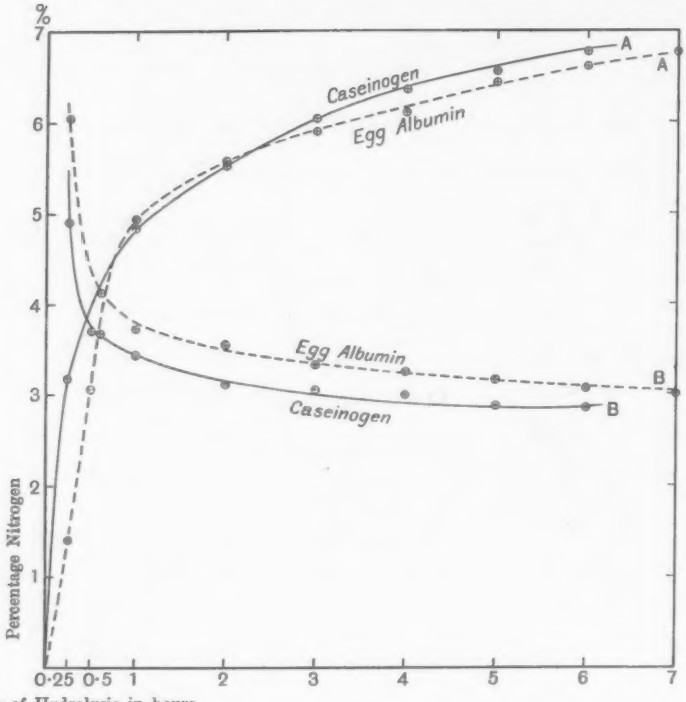

Fig. 2.

Curves showing: A. Rate of Liberation of Amino-Nitrogen.
B. Rate of Liberation of Nitrogen precipitated by Phosphotungstic acid.

IV. *Estimation of Nitrogen precipitated by Phosphotungstic Acid.*

A. *Caseinogen.*

75 cc. of the liquid were used.

Time of hydrolysis in hours	Weight of caseinogen, g.	Number of cc. 0·1 N HCl used (actual titration × 3·33)	Nitrogen equivalent, g.	Percentage nitrogen	Mean of duplicates
0·25	0·9994	35·34	0·04947	4·96	4·900
	1·0613	36·67	0·05131	4·84	
0·50	1·1179	29·17	0·04083	3·66	3·695
	0·9648	25·67	0·03594	3·73	
1	1·0627	26·16	0·03662	3·40	3·440
	1·0769	26·34	0·03687	3·48	
2	0·9842	21·50	0·03010	3·06	3·100
	1·1830	26·50	0·03710	3·14	
3	0·9467	20·83	0·02912	3·08	3·040
	0·9553	20·50	0·02870	3·00	
4	0·9026	19·00	0·02660	2·95	3·010
	0·9896	21·67	0·03034	3·07	
5	0·9373	19·00	0·02660	2·88	2·860
	1·0038	20·67	0·02894	2·84	
6	1·2733	25·67	0·03594	2·84	2·830
	1·0307	20·83	0·02916	2·82	

IV. *Estimation of Nitrogen precipitated by Phosphotungstic Acid.*

B. *Egg albumin.*

Time of hydrolysis in hours	Weight of egg albumin, g.	Number of cc. 0·1 N HCl used (actual titration × 3·33)	Nitrogen equivalent, g.	Percentage nitrogen	Mean of duplicates
0·25	0·9942	42·34	0·05922	5·96	6·055
	1·0085	44·34	0·06202	6·15	
0·50	0·8607	25·67	0·04018	4·17	4·110
	0·9895	28·67	0·03594	4·05	
1	0·8164	21·67	0·03034	3·72	3·735
	1·0576	28·34	0·03966	3·75	
2	0·8806	22·50	0·03150	3·58	3·550
	0·7636	19·16	0·02684	3·52	
3	1·0956	25·67	0·03594	3·28	3·320
	0·9860	23·67	0·03314	3·36	
4	0·7748	18·17	0·02541	3·28	3·260
	1·0865	25·00	0·03500	3·24	
5	1·0181	22·67	0·03174	3·14	3·185
	0·9522	22·00	0·03080	3·23	
6	1·0098	21·34	0·02988	2·96	3·025
	1·0429	23·00	0·03220	3·09	
7	0·9884	21·34	0·02988	3·02	2·990
	0·8228	17·34	0·02428	2·96	

Discussion of results.

I. *Ammonia.* In the very early stages of the hydrolysis the rate of liberation of ammonia is very rapid, and consequently the curves (Fig. 1) rise very steeply. This early period is then followed by one in which the ammonia comes off at a steady slow rate, so that the curves during this second period rise slowly. Finally the amount of ammonia liberated reaches a limit and the curve becomes a straight line parallel to the time axis.

The ammonia liberated on protein hydrolysis is always considered as being combined in the protein in the form of an amide, and Osborne and his fellow workers have shown that the amount of ammonia liberated on complete acid hydrolysis of a protein roughly corresponds to what would be obtained from the half amides of the two dibasic acids, glutamic and aspartic, contained in the protein. The results here obtained are in accord with such an explanation.

The experimental error in the estimation of ammonia is probably rather high, chiefly owing to the smallness of the quantities measured, but still there is no doubt that the curves indicate the rate at which the ammonia is set free.

II. *Humin Nitrogen.* From the mean of all the values obtained it appears that 0·31 per cent. nitrogen in the case of egg albumin and 0·29 per cent. nitrogen in the case of caseinogen is removed by the magnesia.

The values for 0·25 hour's hydrolysis are not included here as evidently in these cases the magnesium oxide carries down with it bodies other than humin substances.

III. *Amino-nitrogen.* The two curves (Fig. 2 *A*) obtained showing the rate of liberation of amino-nitrogen are very similar, although of course by no means identical. Moreover, they resemble in a striking manner the curves obtained for the rate of liberation of amino-nitrogen when the hydrolysis of the protein has been accomplished by means of an enzyme, such as trypsin. Hence the process of hydrolysis is the same whether the hydrolysing agent be a vigorous one, such as hydrochloric acid, or a slow one, such as trypsin. These curves for the amino-nitrogen illustrate very clearly the difference in composition and behaviour of the primary products of hydrolysis of the two proteins. The curves twice intersect, indicating that the rate of liberation of amino-nitrogen from the primary products of hydrolysis of the two proteins, twice changes.

In the first phase of the hydrolysis the amount of amino-nitrogen set free is greater in the case of caseinogen, indicating that more amino-acids and polypeptides are formed during this period in the hydrolysis of caseinogen than in that of egg albumin. In the second phase of the hydrolysis this state of affairs is reversed, showing that during this period the complex polypeptides derived from egg albumin are broken down more readily than those formed in the primary decomposition of caseinogen.

Finally after the second intersection of the curves the rate of liberation of amino-nitrogen is greater again in the case of caseinogen, proving that the simpler polypeptides derived from caseinogen are more easily broken down to amino-acids than those produced from egg albumin. This of course is what one expects when it is remembered that the complete hydrolysis of egg albumin takes longer to accomplish than that of caseinogen.

IV. *Nitrogen precipitated by phosphotungstic acid.*

Here again the two curves (Fig. 2 *B*) are very similar in form, but throughout the whole period investigated the caseinogen curve lies below that of the egg albumin.

In the early part of the hydrolysis there is a large amount of nitrogen in the phosphotungstic acid precipitate in both cases, showing that there

is a good deal of polypeptide nitrogen in the precipitate. Afterwards, however, the phosphotungstic acid contains very little polypeptide nitrogen, for from three hours onwards the curve runs nearly parallel to the time axis, though it does fall just a little.

The big difference between the values of the phosphotungstic acid nitrogen after 0·25 and after 0·5 hour's hydrolysis, is undoubtedly due to the decomposition of complex polypeptides into simpler ones, which are not precipitated by phosphotungstic acid. This point is brought out more clearly by drawing the curve total nitrogen accounted for, against time.

In the figures which follow no account is taken of the fact that the diamino-acids, which are precipitated by phosphotungstic acid, contain a certain amount of amino-nitrogen and hence some of the nitrogen is accounted for twice. This of course has no bearing on the point at issue.

Total Nitrogen accounted for in the above Estimations.

Time of hydrolysis in hours	Percentage ammonia (amide nitrogen)	Percentage amino-nitrogen	Percentage phosphotungstic nitrogen	Percentage humin nitrogen	Total percentage
A. *Caseinogen.*					
0·25	1·340	3·190	4·900	0·63	10·06
0·50	1·360	3·705	3·695	0·29	9·05
1	1·385	4·885	3·440	0·29	10·00
2	1·405	5·525	3·100	0·29	10·33
3	1·440	6·020	3·040	0·29	10·77
4	1·460	6·350	3·010	0·29	11·11
5	1·505	6·565	2·860	0·29	11·22
6	1·495	6·785	2·830	0·29	11·40
B. *Egg albumin.*					
0·25	0·940	1·400	6·050	1·13	9·52
0·50	0·975	3·045	4·110	0·31	8·44
1	1·050	4·930	3·735	0·31	10·02
2	1·100	5·540	3·550	0·31	10·50
3	1·150	5·900	3·320	0·31	10·68
4	1·200	6·100	3·260	0·31	10·87
5	1·245	6·430	3·180	0·31	11·16
6	1·260	6·605	3·025	0·31	11·20
7	1·260	6·765	2·990	0·31	11·32

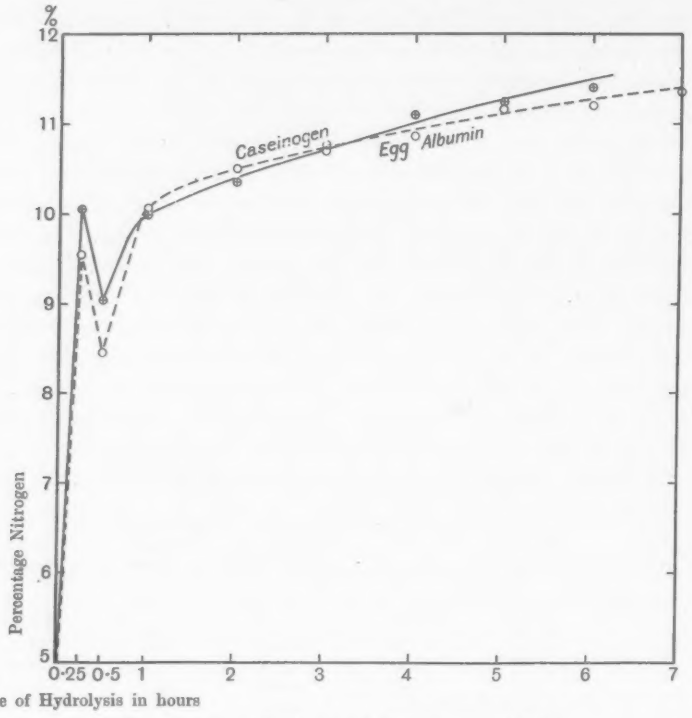

Fig. 3. Curves showing total nitrogen accounted for.

These figures and the curves (Fig. 3) plotted from them show that the total amount of nitrogen accounted for rises steadily with the time except in the case of 0·5 hour, where the total nitrogen accounted for is considerably less than that at 0·25 hour.

This proves definitely that the huge decrease in the amount of nitrogen precipitated by phosphotungstic acid at these two times of hydrolysis, is not nearly accounted for by the increase in the amount of amino-nitrogen found, or in other words it is clear proof that between 0·25 and 0·5 hour's hydrolysis some complex polypeptides which were precipitated by phosphotungstic acid after 0·25 hour's hydrolysis, have been broken down into simpler products, which are not precipitated by this reagent.

The fact that at no time is the whole of the nitrogen of the proteins accounted for shows that there are many polypeptides which are not precipitated by phosphotungstic acid, a point which is further emphasised by

the fact that the phosphotungstic nitrogen curves run nearly parallel to the time axis after a few hours' hydrolysis, i.e. long before the hydrolysis of the protein is nearly complete.

Another point which the phosphotungstic nitrogen curves indicate is that the diamino-acid content of caseinogen is less than that of egg albumin, a result which is in direct opposition to the published figures, viz.:

		Hart [1901] Caseinogen	Hugonenq et Galimard [1906] Egg albumin
Lysine	...	5·80 %	2·15 %
Arginine	...	4·84	2·14
Histidine	...	2·59	0·00

from which figures it appears that caseinogen should have 3·36 per cent. of diamino-nitrogen and egg albumin 1·10 per cent.

Although in the above experiments the hydrolysis was not complete, the curves show that it is impossible to obtain, on complete hydrolysis, results compatible with the published figures, and the indicated differences seem large enough to warrant a re-determination of the diamino acids in both cases.

Summary.

These results prove definitely that many of the simpler polypeptides are not precipitated by phosphotungstic acid, and moreover indicate that a definite point exists at which the more complex peptides are broken down into simpler ones, most of which are not precipitated by phosphotungstic acid, at which therefore it is advantageous to stop the hydrolysis so that the complex polypeptides may be isolated. The isolation of such bodies, and the study of their properties, it is my intention to essay in the near future.

Moreover the experiments here described emphasise the difference in the constitution of caseinogen and egg albumin and the consequent difference in composition of their primary products of hydrolysis.

Finally, I wish to thank Professor T. B. Wood for suggesting this work, and to say how much I appreciate the interest he has shown in its progress.

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XXIV. DIAGRAMMATIC CO-ORDINATION OF PHENOMENA RELATING TO AGGREGATION OF SOLS.

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The published investigations of the following phenomena embrace a considerable portion of the facts so far accumulated on the causes inhibiting or actuating the aggregation of sols, especially when they are mixed with proteins and electrolytes:

1. *The action of electrolytes on sols*—causing the running together of sol particles when added to the sol in sufficient concentration.

2. "*Protection*"—the property of proteins and certain other substances in preventing the aggregation of sols by electrolytes.

3. *Change of sign of the electrostatic charge on the protected sol particle.*

4. *Non-coincidence of point of maximum flocculation and point where sign of observed particle changes.*

5. *Change of sign of charge on the protein at its isoelectric point.*

6. *Mutual precipitation of dissimilarly charged colloids* in solutions free from electrolytes, including the particular case where one of the colloids is amphoteric and has the properties of "protection," e.g. is of a protein nature; also the solubility of the precipitate formed in excess of either constituent.

7. "*Irregular series*" (Bechold) and "*Pre-zone phenomenon*" (Buxton).

8. "*Reversible and irreversible aggregation.*"

Up to the present these phenomena have been generally investigated one at a time. Each worker has naturally chosen experimental conditions particularly suitable for the study of one phenomenon only. Little or no co-ordinated work has been done, linking up on a quantitative basis the known facts, and demonstrating them as parts of a well-ordered scheme of things, easy to remember, and, on the surface, easy to understand.

The plotting of results, obtained in this class of work, in chart form, undertaken primarily for my own use, has proved so helpful to me in

following out these relationships that I feel in a position to recommend them for general use in expressing the results of experiments of this type.

In working with a very finely divided oil emulsion, the particles of which were so small that, without misapplying the term, it could be called an "oil sol," I found, in common with previous experience, that the addition of hydrochloric acid in sufficient strength caused the particles to run together and the oil to separate. The presence of a sufficient quantity of gelatin, however, prevented this from happening; the sol was "protected" in this case. When the gelatin was in certain concentrations, not too small and not too great, the oil particles aggregated immediately if just the right amount of acid was added, but not if the acid was too strong or too weak.

This seemed to point to some new and peculiar phenomenon where the gelatin acted as an "activator" to the acid, for in this case the acid was far too weak to aggregate the sol alone. It was not till later that it was seen that this fitted into the general scheme of things and was capable of expression in terms of things known, and that the invention of a new "phenomenon" and a corresponding vocabulary was unnecessary. In other cases where gelatin of a certain strength caused immediate aggregation of the oil in the presence of a small quantity of acid incapable of doing this alone, it was found that all strengths of acid greater than this brought about the same result.

To simplify the matter of making and studying these mixtures it was decided that each should contain 2 cc. of oil sol, 2 cc. of gelatin solution and 2 cc. of hydrochloric acid solution. Rows of these mixtures were put up in test tubes. In any one row the 2 cc. of acid placed in each tube was of the same strength throughout; the concentration of the 2 cc. of gelatin solution added varied from tube to tube. The strength of acid used was changed from row to row.

As very dilute solutions were sometimes used it was found necessary from the outset to express their concentrations as powers of ten, either of actual concentration or normality. For instance the strength of a gelatin solution was expressed in terms of its concentration. A 1 in 10 solution was expressed as "gelatin 10^{-1}," for example, and a 1 in 15,000 solution as $10^{-4\cdot2}$. Similarly 0·001 N hydrochloric acid was written 10^{-3} N HCl.

After the mixtures had been made two hours they were examined carefully, and notes made of those which showed to the eye no change of state of the sol, those in which partial aggregation had occurred, and those in which the separation of the oil was complete.

Observations in the electric field using the microscope method showed

that the oil particles in some of these mixtures were positively and in others negatively charged. The sign of the charge did not depend upon the state of aggregation—either charge was observed in tubes containing mixtures which did not aggregate just as in those that did.

When the results of these two sets of observations were expressed in tabular form little that was intelligible could be made of them; in the form of a chart they are all summarised in Fig. 1.

Aggregation Diagram No. 1.

It will be remembered that each mixture consisted of 2 cc. of oil sol, 2 cc. of gelatin solution and 2 cc. of hydrochloric acid. As ordinates are expressed negative exponents of normality of the hydrochloric acid put into the tube; as abscissae the negative exponent of the concentration of gelatin. The tubes were considered one at a time and a mark plotted on the chart at the point corresponding with the concentration of acid and gelatin in the tube indicating (1) that no change had taken place in the distribution of

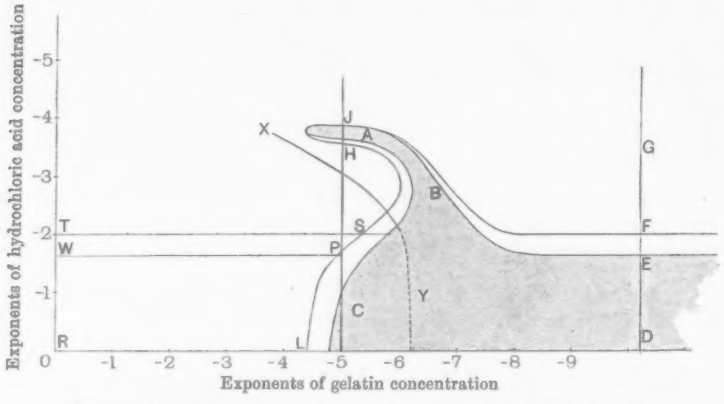

Fig. 1. Aggregation diagram No. 1. Oil sol-gelatin-hydrochloric acid.

the oil particles, (2) that complete aggregation and separation of the oil had occurred, or (3) that aggregation was only partial.

The sign of the oil particles, whether aggregated or not, was indicated by a small + or −. Lines were now drawn on the diagram by the help of these distinguishing marks. The first, *LHJF*, enclosed all points where aggregation, whether partial or complete, was observed. Another, *CABE*, enclosed all points where separation of the oil was complete.

The line *XY* was drawn through all points where the oil particles appeared to have no electric charge; that is, they did not wander in the electric field. In all mixtures represented by points to the right of this line, the particles were negatively charged. All points to the left of this line correspond to mixtures in which particles were positively charged.

The chart—called for convenience an "aggregation diagram"—represents the state of aggregation and sign of the dispersed particle, two hours after mixing, of every possible mixture of equal volumes of oil sol, gelatin solution and hydrochloric acid. Mixtures stronger in HCl than 0·33 normal were not plotted in the present instance simply to avoid unnecessary complication of the diagram.

At this stage it will be well to examine this chart with reference to the list of phenomena given at the beginning of this paper.

1. *The action of electrolytes on sols.* The action of electrolytes in the absence of protein is seen in the extreme right of the diagram. In the particular case shown in Fig. 1 the protein in a dilution of one in one hundred million or more has no effect. The lines bounding the aggregation area become parallel to the abscissae, and may be produced to infinity to the point where the protein is infinitely dilute. The ordinate *DEFG* represents, therefore, the behaviour of mixtures of 2 cc. sol, 2 cc. water and 2 cc. of every strength of electrolyte solution, in this case HCl. No aggregation occurs with extremely dilute acid, but when the strength of acid used is 10^{-2} N a slight separation of the sol occurs. As the strength of acid increases the amount of sol separation increases until at $10^{-1\cdot6}$ N and all concentrations above this it is described as "complete."

In the case of an electronegative sol and an electrolyte with a polyvalent cation the ordinate *DEF* would be much shorter—a result obtained equally if the sol were electropositive and the electrolyte contained a polyvalent anion.

At present the actual mechanism of electrolyte precipitation of sols is not very well understood. It is hoped that in the course of this work some phenomena may be observed which can be co-ordinated with the facts already known about the conditions determining the stability or otherwise of sols.

2. "*Protection.*" Any point in the area *WPLR* corresponds to a mixture in which the gelatin has prevented the acid from aggregating the oil sol. Enough acid is present to bring this about completely if the gelatin were not present. It may therefore be referred to as the "area of complete protection against aggregation." In the same way the area *WPST* is "the area of complete protection against partial aggregation," for in the mixtures

the behaviour of which is represented by points in this area the acid alone would only partially aggregate the sol in two hours.

Areas of "partial protection against partial aggregation" and "partial protection against complete aggregation" are to be found in the diagram and may some day become matters of study.

3. *The change of sign of the "protected" sol particle.* It is interesting to note that the "area of protection" lies entirely inside the area where the sign of the sol particles, or rather the sol-gelatin complexes, is positive. Also, and this is shown better in Fig. 2 where mastic sol was used, it is possible to have a sol protected by gelatin in a mixture containing as much as one-third of its volume of N HCl, while a mixture exactly similar except that the acid is 6000 times more dilute, flocculates immediately. A result of this kind was observed by Mines [1912] using a gold sol.

4. *Non-coincidence of point of maximum flocculation and point where the sign of the observed particle changes.* The type of flocculation which may be observed in extremely dilute acid concentration if the concentration of the protein be adjusted with great care, is remarkable in that no change of sign of the electronegative sol particle necessarily takes place.

In Fig. 1 the ordinate *CPHJ* passes through points representing a series of mixtures in which are placed say 2 cc. of oil sol, 2 cc. gelatin 10^{-5}, and 2 cc. of hydrochloric acid decreasing in concentration from tube to tube. From normal acid to decinormal acid (10^{-1}) complete flocculation is observed, from there to 10^{-17} the flocculation is incomplete, and as far as can be made out in solutions containing so much electrolyte the sign of the visible particles is +. With acid strengths from 10^{-17} to 10^{-37} there is no change whatever visible in the state of aggregation of the sol, but at HCl 10^{-3} N no charge can be detected on the particles, while at 10^{-37} and all tubes following, which contain weaker acid, the sign of the particles is −. Following on the series; partial separation is observed with acid 10^{-36}; at 10^{-37} and 10^{-38} the separation is complete; at 10^{-39}, 10^{-4} and onwards no change in sol aggregation can be seen.

The change of sign of the sol particles with constant gelatin concentration and diminishing acid takes place outside either of the ranges where aggregation occurs; and this assumes a particular interest when it is remembered that globulin suspensions are negatively charged at the point of maximum flocculation, and on increasing the acid concentration they are well dispersed before the visible particles are electrically neutral. This observation recorded by Chick [1913] I have confirmed. The figures given in the paper referred to are $H^{\cdot}$ concentration $32 \cdot 10^{-7}$ for the point of maximum flocculation [cf.

Michaelis and Rona, 1910]. At $H^{\cdot}$ concentration $748 \cdot 10^{-7}$ the sign is still −, changing to + at $1140 \cdot 10^{-7}$.

5. *Change of sign of the protein at its isoelectric point.* A line may be drawn through all points having $H^{\cdot}$ ion concentration equal to the isoelectric point of the protein. This will divide the diagram into two parts representing mixtures containing + charged and − charged protein respectively.

6. *Mutual precipitation of dissimilarly charged colloids.* Biltz [1904] in his classical confirmation of earlier work by Picton and Linder [1897], and Lottermoser [1901] expressly state that *in the absence of electrolytes* oppositely charged colloidal solutions precipitate one another from solution when mixed in certain definite proportions, and that the precipitate is soluble in excess of either constituent. Whether this limiting condition is ever fulfilled in practice is questionable; but still the mixtures containing only hydrochloric acid $1/3 \cdot 10^{-3}$ N HCl may be considered in the class of mixtures of colloidal solutions to which Biltz referred. Following the abscissa representing a row of mixtures made by mixing say 2 cc. acid 10^{-3} N, 2 cc. oil sol and 2 cc. gelatin solution, decreasing in concentration from tube to tube, it is seen that mutual precipitation only occurs within a certain range of gelatin-hydrosol ratios. The large number of the variables from tube to tube, however, all of which affect aggregation, makes the analysis of this phenomenon in the present state of our knowledge very difficult, and postpones the complete understanding of the curious shape of the aggregation area.

7. "*Irregular Series,*" and "*Pre-zone phenomenon.*" The first of these terms was used by Bechold [1904] to describe the results obtained by aggregating various sols and bacterial suspensions with solutions of ferric and aluminium chlorides. Buxton and Rahe [1910] saw in these experiments the demonstration of the action of the acid solution when concentrated, and the precipitating action of a colloidal solution in greater dilutions. In order to avoid confusion they referred to their numerous flocculation experiments in which irregular series occurred from other causes as demonstrations of the "pre-zone phenomenon."

8. *Reversible and Irreversible Aggregation.* Sols such as gold, oil and mastic are referred to as irreversible hydrosols because when flocculated by electrolytes they do not disperse again when the electrolytes are removed by dialysis, and, when flocculated by traces of acid, the reversal of this effect is not obtained by addition of corresponding traces of alkali.

In the aggregation area of Fig. 1 there may be recognised two types of flocculation. To the extreme right, where separation of the sol has occurred

in the absence of protein, the aggregation is not reversible by adding alkali. At *JH* where the protein plays an all-important *rôle* in the flocculation, the addition of a trace of alkali results at once in the almost complete reversal of the phenomenon. In other parts of the aggregation area it would appear as if both the reversible and irreversible type of aggregation were found varying in relative amount with the position on the diagram. When the sol is of oil the former type shows a grape bunch appearance under the ultramicroscope, while the latter aggregates are single drops of oil formed by the coalescence of the minute particles. With gold sol-gelatin-HCl mixtures both types are also readily observed.

With mastic-gelatin-HCl mixtures these phenomena may be observed with even greater readiness. If care be taken to adjust the proportions so that aggregation occurs with a maximal quantity of gelatin and traces only of hydrochloric acid, the electronegative aggregates are, as would be expected from the diagram, dispersed by traces of alkali or acid. The reversibility of the flocculation diminishes on standing just as globulin suspensions after a time become insoluble. When in addition it is found that the flocculae are dispersed and not, as would be expected, aggregated further by the addition of a small quantity of sodium chloride, the analogy to euglobulin appears so striking that the possibility of its being a complex physically comparable with the mastic-gelatin or oil-gelatin complexes here dealt with cannot be overlooked[1].

Aggregation Diagrams Nos. 2, 3 and 4 a.

After this preliminary description of the method of investigation pursued in coordinating various phenomena connected with the aggregation of sol-protein complexes, three particular experiments may be considered. These refer to the behaviour of mixtures of equal volumes of a particular *mastic* sol, gelatin and an electrolyte solution, and may be called for convenience experiments 2, 3 and 4. This particular sol I found more easy to work with than an oil sol, for the various stages of aggregation from a slight clouding of the blue opalescence in twenty-four hours to an immediate and complete flocculation of the whole of the material could be picked out more or less accurately and recorded.

The difference between the three experiments lay in the nature of the

[1] The flocculated complex made from pseudoglobulin (horse) and a sol of the lipoid from euglobulin (horse) resembles euglobulin still more closely. Even after several days' standing it is instantly and completely dispersed by traces of alkali or acid.

electrolyte constituents of the mixtures. In all three experiments the electrolyte varying in amount from row to row was hydrochloric acid. In experiment 3, however, every mixture was in addition 0·011 N with respect to sodium chloride: in experiment 4, every mixture was in addition 0·011 N with respect to sodium acetate. When the mixtures had been prepared and

Mastic-gelatin-electrolyte Aggregation Diagrams.

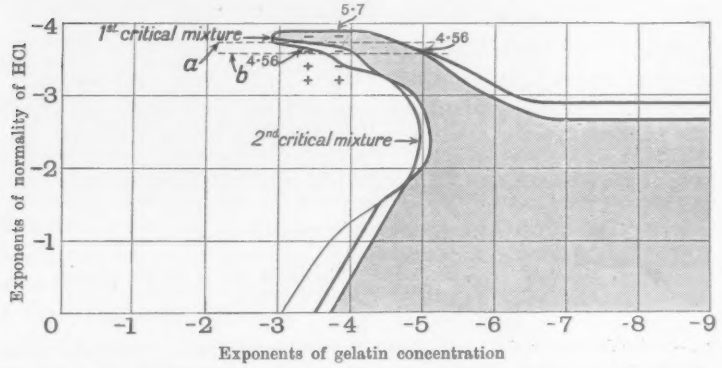

Fig. 2. Aggregation diagram No. 2. Electrolyte HCl in varying amounts.

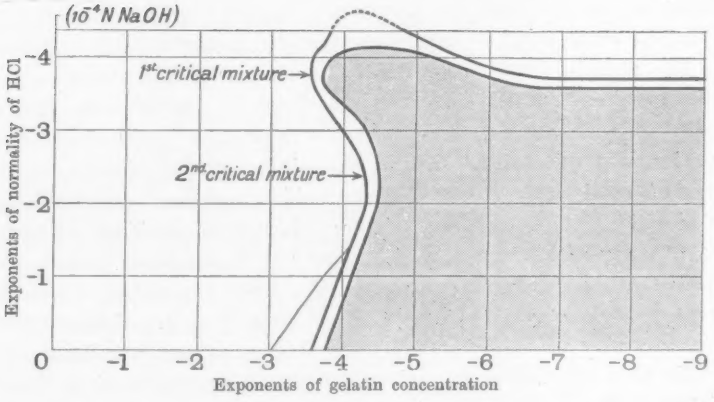

Fig. 3. Aggregation diagram No. 3. Electrolyte HCl in varying amounts +0·033 N sodium chloride.

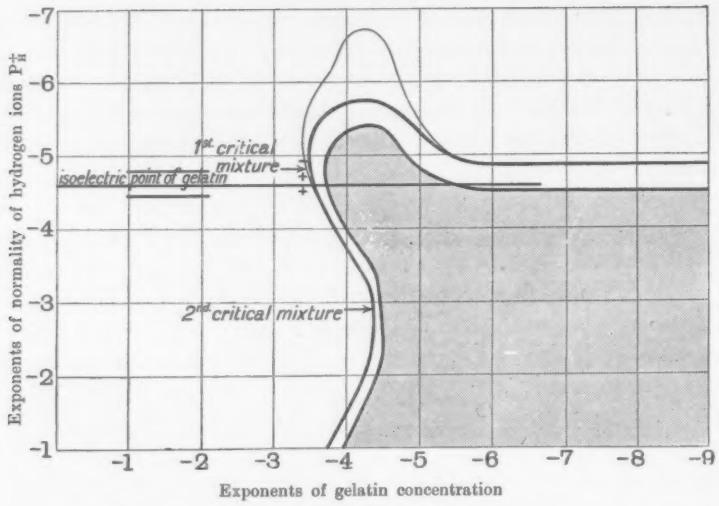

Fig. 4. Aggregation diagram No. 4A. Electrolyte HCl in varying amounts +0·033 N sodium acetate.

NOTE.—Each mixture contains equal volumes of the three constituents. The concentrations of gelatin and HCl whose exponents are plotted in these diagrams are the concentrations used to make the mixtures. To find the actual concentration in a mixture add log 3 = ·477 to the negative exponent. In Fig. 4 the $H^{\cdot}$ ion concentrations, plotted as ordinates, refer to the $H^{\cdot}$ ion concentrations of the mixtures.

allowed to stand 24 hours, one aggregation diagram for each experiment was plotted in which negative exponents of acid concentration were ordinates and the negative exponents of the gelatin concentration abscissae. The sign of the electrostatic charge on the observable particles was also recorded where expedient. The diagrams from experiments 2 and 3 are reproduced in this paper. In each the stippled area represents complete flocculation: the space between the two thick lines just outside this area includes all points representing mixtures which show considerable aggregation of the particles in twenty-four hours but do not leave perfectly clear supernatant fluid above the flocculae. The areas bounded by a thin line represent slight aggregation only. The diagram from experiment 4 which is not necessary for discussion of these results has not been reproduced.

The next step was to determine the hydrogen ion concentration of every mixture in the three experiments.

In experiments 2 and 3, the rows containing acid less than $10^{-3\cdot4}$ N

presented very considerable difficulties. The reaction inertia and the hydrogen ion concentration were low, and in those tubes to which no sodium chloride was added the conductivity was also low. By working with sufficient care accurate results were obtained in tubes where no flocculation had occurred but in the remaining tubes the results were of such a nature that they are best considered separately and recorded with certain reservations.

In experiment 4 the mixtures all lent themselves readily to accurate P_H^+ determinations and so a second aggregation diagram could be prepared in which the ordinates were the negative exponents of hydrogen ion concentration. This diagram 4A has been reproduced (Fig. 4). A comparative study of this diagram and the two preceding is instructive.

It is possible to regard diagram 3 as being derived from diagram 4A by submitting the ordinates to a process similar to that of opening out a telescope, the telescope having a large number of joints which do not slide out necessarily at the same rate. This suggests that if the influence of changing hydrogen ion concentration be ignored, sodium chloride and sodium acetate in strength 0·011 N behave very similarly in relation to the flocculation of mastic-gelatin mixtures. A similar relationship between diagrams 2 and 4A does not hold.

Hydrogen ion determinations for certain important mixtures in experiments 2 and 3 were made quite satisfactorily and a few are recorded on the diagram 2, but in view of the labour involved, corresponding diagrams, 2A and 3A, in which the ordinates would have been hydrogen ion concentrations, could not be prepared complete for the present paper (see p. 182).

In experiment 4 it was found that throughout each row of mixtures the $H^{\cdot}$ ion concentration did not vary appreciably, that is to say, the mixtures along the row were *isoxyntic* (i.e. of equal $H^{\cdot}$ ion concentration) in spite of a variation in gelatin content from $10^{-3\cdot48}$ to infinite dilution. At this stage certain relationships can be established by comparing diagrams 2, 3 and 4A.

(1) *Relationships between composition and possibility of flocculation.*

(*a*) In each of the three experiments there is one mixture which is just on the verge of flocculation and in which flocculation would not have occurred at all if the gelatin concentration had been a little greater. Neither would it have flocculated if a little more acid or a little less acid had entered into its composition. Its reaction is invariably in the region of the isoelectric point of gelatin. It may be conveniently called the "1st critical mixture" of the system mastic-gelatin-electrolyte. In experiment 2 the "1st critical mixture" contains five times as much gelatin as in experiments 3 and 4.

(*b*) It is seen that at the upper limits of HCl concentration flocculation is not appreciably influenced by the presence of 0·011 N NaCl. The effect of this concentration of sodium chloride is shown markedly, however, when the minimal concentrations of hydrochloric acid at which complete flocculation of mastic alone can be observed are examined.

In experiment 2 if the mixture made from HCl $10^{-2\cdot4}$ N, gelatin $10^{-5\cdot0}$ and mastic be considered it is seen that partial flocculation has occurred. If stronger acid with the same gelatin solution, or weaker acid with the same gelatin solution, or weaker gelatin with the same acid strength, were used to make the mixture flocculation would be complete.

Similarly in experiment 3 if the mixture made from HCl $10^{-2\cdot4}$ N, gelatin $10^{-4\cdot4}$ and mastic be considered the same fact is evident. More acid, or less acid, or less gelatin, other constituents of the mixture being left unaltered, will give mixtures flocculating to the full extent. And, again, in experiment 4 there is one mixture made from gelatin $10^{-4\cdot4}$ to which the same considerations apply.

The three mixtures, one in each experiment, which have this curious relationship to those contiguous to them may also be called "critical mixtures." To distinguish them from those of the type described in (*a*) they may be called "2nd critical mixtures."

Critical mixtures whether "1st" or "2nd" have this in common—a slight increase in gelatin concentration would inhibit flocculation completely. They differ in that a "1st critical mixture" is not flocculated by more acid or less acid, while a "2nd critical mixture" is.

The three "2nd critical mixtures" considered—one from each of the experiments 2, 3 and 4—were found experimentally to have the same hydrogen ion concentration $P_H^+ = 2\cdot9$.

They are made from gelatin $10^{-5\cdot0}$ when electrolyte is not added and $10^{-4\cdot4}$ when sodium chloride or acetate is present, i.e. those which are 0·011 N with respect to sodium salt contain four times as much gelatin as the other. It is suggested that the increased thickness of coating of gelatin (at $P_H^+ = 2\cdot9$ in all three cases) resulting from the inclusion of four times as much gelatin per unit volume in the mixture, balances the flocculation effect of 0·011 N sodium ions. On p. 186 an explanation of the existence of the "2nd critical mixture" has been attempted.

In Figures 2 and 3 it is seen that as the acid concentrations increase towards the foot of the diagram, the effect of the added sodium chloride in experiment 3 becomes less and less noticeable. The apparent discrepancy of diagram 4A in this respect disappears when the nature of the ordinates

is recognised. Diagrams 2A, 3A (not reproduced) and 4A are identical towards the base line—$P_H^+ = 10^{-9}$ (normal $H^{\cdot}$ ion concentration).

Relationships between flocculation area and (a) the isoxyntic line corresponding to the isoelectric point of gelatin; (b) the line passing through all points where the observed particles are electrically neutral.

Experiment 4.

In experiment 4 the following determinations were made with a transport apparatus similar in design to that used by Michaelis [1909]. It was made of glass of one cm. bore all in one piece. Rubber was dispensed with entirely. Non-polarisable electrodes, Cu in $CuCl_2$, and Ag in N NaCl, were employed, and many test experiments were made which left no doubt as to the accuracy of the results.

Fluid in side tubes—32 cc. water + 30 cc. acetate mixture + 30 cc. 5 per cent. alcohol.
Fluid in middle tube—30 cc. gelatin $10^{-3.6}$ + 30 cc. acetate mixture + 30 cc. mastic sol.

Composition of acetate mixture	P_H^+ of middle tube	Direction of migration	
3·3 cc. N sod. acetate + 2·2 cc. N HCl + water to 100 cc.	4·28	+	Isoelectric
,, ,, 2·0 ,, ,, ,,	4·47	+	point of
,, ,, 2·0 ,, ,, ,,	4·52	+	gelatin
,, ,, 1·5 ,, ,, ,,	4·75	+	4·6
,, ,, 1·2 ,, ,, ,,	4·92	−	(Michaelis)
,, ,, 1·2 ,, ,, ,,	4·92	−	
,, ,, 1·0 ,, ,, ,,	5·05	−	

The meniscus of the moving mastic suspension remained sharp and could be observed with great accuracy. After about an hour the direction could be ascertained definitely even in the solutions $P_H^+ = 4{\cdot}75$ and $P_H^+ = 4{\cdot}92$. Gelatin concentration $10^{-3.6}$ was used throughout these determinations because it was the weakest solution that could be used without involving flocculation.

On the diagram + and − signs represent the results of some of the determinations just described. It is remarkable to note that the observed particles in the 1st critical mixture are almost without any electric charge. The isoelectric point of gelatin is possibly slightly more acid than this, indicating that the uncharged particles result from a combination of neutral or possibly slightly negative gelatin and the electronegative nucleus.

Experiment 2.

In the 1st critical mixture of experiment 2, in which electrolytes were excluded as far as possible, the effect of the electronegative nucleus on the charge of the mastic-gelatin complex will be seen to be much more marked.

As previously stated a considerable time was spent in determining under the best experimental conditions the hydrogen ion concentrations of certain mixtures in experiment 2 which had not flocculated. In every case a series of mixtures was made simultaneously in which the position of the flocculation area was verified and then the non-flocculated mixtures of this same series examined with respect to their hydrogen ion concentration and the sign of the electrostatic charge of the observable particles by the microscope method. The mixtures were prepared and the observations repeated many times with concordant results.

Flocculated mixtures were also examined in the same way. The particles were invariably electronegative but the hydrogen ion concentrations did not furnish results sufficiently trustworthy to be considered in this connection.

The results of the observations are given below. Some are recorded on the diagram.

Mixtures of equal volumes of mastic sol, gelatin solution and HCl taken from Exp. 2.

Exponent of gelatin concentration used	Exponent of normality of HCl used	E.M.F.	P_H^+	Sign of observed particle
−3·6	−3·0	0·455	3·53	+
,,	−3·4	0·490 : 0·488	4·12	±
,,	−3·6	0·515 : 0·515	4·56	−
,,	−3·9	0·580	5·70	−
−4·0	−3·0	0·455	3·54	+
,,	−3·4	0·491 : 0·485	4·10	−
,,	−3·6	0·502 : 0·507	4·40	−
,,	−4·0	0·534 : 0·544	5·00	−
−4·4	−3·0	0·455	3·54	+
,,	−3·4	0·492	4·18	−
−5·0	−3·5	0·513	4·54	−
,,	−3·6	0·515	4·56	−

From the evidence of these figures the following statements may be made:

(*a*) In the "horn" like part of the flocculation area the observed particles are negatively charged. In mixtures, just below this, which have not flocculated, the particles are also negatively charged. Comparison with aggregation diagram No. 1 where an oil sol was used shows that the

line XY passing through all mixtures where the observed particles of the diagram are electrically neutral does not pass so close to the "horn" like part of the flocculation area as in diagram 2. The other striking difference between the two diagrams is the relative stability of the two sols to $H^{\cdot}$ ion concentration. It is suggested that these two phenomena have a common factor.

(*b*) *Passing upwards through the "horn"* along a series of mixtures made from gelatin solution of concentration $10^{-3\cdot6}$ *the* $H^{\cdot}$ *ion concentration is changing very rapidly,* viz. from $P_H^+ = 4\cdot56$ at acid $10^{-3\cdot6}$ N to $P_H^+ = 5\cdot70$ at acid $10^{-3\cdot9}$.

It is therefore established that the isoxyntic lines P_H^+ 4·45 and P_H^+ 4·80 representing the upper and lower limits of the isoelectric range of gelatin as measured by Michaelis, either both pass below the "horn" or that the upper P_H^+ line 4·8 passes through the lower part of the "horn."

The precise positions of these lines cannot be determined with the same accuracy as in experiment 4, so that they are represented by dotted lines in the diagram labelled *a* and *b*.

(*c*) Mixtures which, if the available data with regard to the isoelectric point of gelatin be correct, would show migration of gelatin to the cathode nevertheless contain observable particles carrying a definite negative charge.

These results from experiments 4 and 2 may be summarised as follows:

It is established, within the limits of experimental error, that, in the presence of 0·011 N sodium acetate, the 1st critical mixture contains electrically neutral particles, and has the hydrogen ion concentration at which no wandering of gelatin can be detected in transport experiments. The sign of the particle in this and contiguous mixtures is, therefore, that of its coating: the electronegative character of the mastic nucleus does not manifest itself at all.

In the absence of electrolytes except minimal traces of HCl, and gelatin of such concentration that when the HCl content is increased the line representing the series of mixtures passes downwards through the middle of the "horn" like projection of the aggregation area, the above coincidences do not occur. Instead the following phenomena occur in the following order:

(1) No aggregation; gelatin negative; all observable particles negative.

(2) Aggregation; gelatin negative; all observable particles negative.

(3) Aggregation; gelatin sign not discoverable by transport experiments; all observable particles negative.

(4) No aggregation; gelatin positive; all observable particles negative.

(5) No aggregation; gelatin positive; all observable particles positive.

The isoelectric range of gelatin was determined in the presence of sodium acetate and hence the above results, in so far as they depend upon this determination, are open to discussion. It is extremely probable, however, that, in the absence of added electrolyte, the electrically neutral particle is made up of an electropositive gelatin coating and an electronegative mastic nucleus. On the other hand it may be stated definitely that the particles in the 1st critical mixture and all other mixtures represented by points in the "horn" like part of the aggregation area are electronegative. All mixtures containing electrically neutral particles are relatively more acid, and show no sign of aggregation.

I can offer no explanation of the results. Every endeavour has been made to discover faults in the observations.

Liberation of alkali from the glass of the microscopic cell was at first considered a possible source of error, but a considerable number of experiments showed that this did not account for the effects observed.

Theoretical discussion of the observed results.

The existence of the "horn" in Figs. 1 and 2, or rather its suppression in Figs. 3 and 4, due to the presence of 0·011 N sodium chloride or sodium acetate, is the manifestation of a phenomenon the explanation of which may for the present be referred to the conjectures concerning the salt dispersion of globulin [Hardy, 1905; Mellanby, 1905; Chick, 1913], denaturated serum protein [Chick and Martin, 1912] and methylimino compounds [Schryver, 1910].

Since, in experiments 3 and 4, 0·011 N sodium salt was present, while it was not present in experiment 2, the series containing gelatin $10^{-5\cdot0}$ in experiment 2 is taken as being comparable with those containing $10^{-4\cdot4}$ in experiments 3 and 4. The reasons for this are stated on p. 180. These series are now to be considered first of all independently, and then with regard to their mutual relationship.

Experiment 2. *Series of mixtures made from gelatin* 10^{-5} *and hydrochloric acid of increasing concentration.*

The facts observed are that with acid from infinite dilution to $10^{-3\cdot7}$ no flocculation occurs; with acid $10^{-3\cdot6}$ there is a partial flocculation; with all stronger acids flocculation occurs except with one strength of acid, namely,

$10^{-2\cdot4}$ N, which is on the verge of flocculation, and is the "2nd critical mixture."

Also, the P_H^+ of the mixture which is on the verge of flocculation made from $10^{-2\cdot6}$ acid is 4·56, and the observed particles of this mixture and all the neighbouring mixtures are negatively charged. It will be remembered that the isoelectric range of gelatin is $10^{-4\cdot45}$ to $10^{-4\cdot8}$.

Among the phenomena known to be concerned in this flocculation or non-flocculation are the surface tension effects at the surfaces of the particles. These are partly of an electrical nature with purely surface effects as concomitant factors. Practically the whole of the gelatin present is in this series on the surfaces of the mastic particles—a point probably capable of demonstration by differential filtration experiments. The series may be divided into two parts. The first consisting of mixtures containing acid from infinite dilution to say $10^{-2\cdot9}$ N HCl and the latter from this strength to normal acid.

In the first part of the series it is known that hydrogen ions are not present in sufficient quantity to flocculate mastic at all even when not coated with gelatin.

This factor can therefore be put aside for the moment and purely surface effects—electrical and otherwise—considered. Gelatin is present in the solution in three forms—just as is any other electrolyte. In accordance with the mathematical investigations of Michaelis and others, the proportion of it which is un-ionised is a maximum at its isoelectric point, and the proportions which are negatively charged and positively charged respectively are equal in amount. If the solution be made more acid the concentration of positively charged gelatin increases. On the alkaline side of the isoelectric point electropositive gelatin ions also exist in concentrations, compared with the total amount of the gelatin present, diminishing with diminishing acidity.

Their existence is necessitated on theoretical grounds but they cannot be detected by the direct transport method. Is it not possible that the flocculation observed on the alkaline side of the isoelectric range established by transport observations is due to traces of electropositive gelatin—and, in fact, that the flocculation of mastic is a very sensitive indicator of gelatin in this condition? The particles are observed to be electronegative—the effect of the electronegative mastic nucleus is not abolished—but it is suggested that the tendency to minimise the surface energy of the system through flocculation becomes operative in spite of electrostatic repulsion when this repulsion is diminished through the acquisition by the particle

of a film of gelatin originally electropositive. The behaviour of the series of mixtures all made from gelatin 10^{-5} and HCl acid from infinite dilution to $10^{-2\cdot9}$ N is seen to be in accordance with the above hypothesis. Continuing the same series from acid $10^{-2\cdot9}$ N to normal acid the phenomenon of the "2nd critical mixture" is observed.

Throughout these mixtures purely surface effects may be taken as being overwhelmed by phenomena of an electrical nature. There are two factors involved. The *first*, which did not come into consideration over the first half of the series, is the flocculating effect of the hydrogen ions on the gelatin-coated mastic particles. The *second* is a factor having the opposite effect—the increasing electropositiveness of the coat, a quantity which may be referred directly to gelatin cation concentration. Adopting the nomenclature used by Sörensen [1912] and one of the equations deduced from fundamental principles quoted by him—

$$\rho = \frac{1}{1 + \frac{k_a}{H^{\cdot}} + \frac{k_b H^{\cdot}}{k_w}},$$

an expression may be obtained giving the rate of change of gelatin cation concentration at any $H^{\cdot}$ ion concentration,

$$A^{\cdot} = \frac{k_b x}{\partial H^{\cdot}} = \frac{k_b A \rho}{\partial H^{\cdot}} = \frac{k_b A \rho H^{\cdot}}{k_w}$$

$$= \frac{k_b A H^{\cdot}}{k_w \left(1 + \frac{k_a}{H^{\cdot}} + \frac{k_b H^{\cdot}}{k_w}\right)}.$$

Differentiating with respect to $H^{\cdot}$,

$$\frac{dA^{\cdot}}{dH^{\cdot}} = \frac{k_w k_b A H^{\cdot} (H^{\cdot} + 2k_a)}{(k_w k_a + k_w H^{\cdot} + k_b H^{\cdot 2})^2}.$$

Putting the dissociation constants and A, the total gelatin concentration, equal to unity and substituting for $H^{\cdot}$ 0·01, 0·10, 0·20, 0·25, 0·33, 0·50, 1·00, 1·50, 2, 10, the values for the expression are 0·02, 0·19, 0·28, 0·32, 0·37, 0·41, 0·33, 0·19, 0·16, 0·01.

From these figures it is seen that the rate of change of gelatin cation concentration increases and then diminishes again with increasing $H^{\cdot}$ ion concentration from its isoelectric point. On the other hand, the rate of change of the tendency to flocculate, that is the factor above, probably increases steadily when referred to the same variable.

The actual result is the algebraic sum of these two factors and it follows from the above relationship that there is a certain $H^{\cdot}$ ion concentration where this algebraic sum is a minimum. This I conceive to be the case

in the mixture which contains acid $10^{-2\cdot4}$ N, and which I have called the "2nd critical mixture."

Experiments 3 *and* 4. *Series of mixtures made from gelatin* $10^{-4\cdot4}$ *with addition of sodium chloride* + HCl, *and sodium acetate* + HCl *respectively. Hydrogen ion concentration increases from each mixture to the next.*

Throughout the series in experiment 4 the same general phenomena are observed with one important difference. The range of hydrogen ion concentration over which flocculation is observed extends to $P_H^+ = 6{\cdot}56$. In Fig. 2 flocculation ceases before the value of hydrogen ion concentration falls to $P_H^+ = 4{\cdot}56$ at the gelatin concentration 10^{-5} which is taken as comparable; and at $P_H^+ = 5{\cdot}7$ under the peculiar circumstances of the series with gelatin $10^{-2\cdot6}$. The addition of 0·011 N sodium acetate makes all this difference. In similar fashion in Fig. 3 complete flocculation was observed in tubes to which no acid at all was added, and almost complete flocculation in tubes containing 10^{-4} NaOH.

If this result of added electrolyte is taken as indicating a widening of the isoelectric range it is comparable with Michaelis and Davidsohn's observations on the isoelectric point of oxyhaemoglobin. In transport experiments they showed [1912] that though the position of the P_H^+ range over which no definite inference could be drawn as to the direction of wandering remained unchanged, its upper and lower limits widened with increasing electrolyte addition.

Proceeding along these two series "2nd critical mixtures" are again observed at $P_H^+ = 2{\cdot}9$. The explanation is probably the same in all three cases even if that put forward is not correct.

Experimental details.

Materials used. The electronegative sols used were of oil, mastic and gold. The *oil sol* found most convenient was the result of many trials and was prepared in the following manner. To 94 cc. of Kahlbaum's acetone, 6 cc. of water are added and 20 cc. of olive oil "sublime." After thorough shaking and settling 40 cc. of the acetone layer are blown in from a pipette as quickly as possible into 1000 cc. of "conductivity" water. The rapidity of mixing of the acetone solution of oil and the water is a contributing factor to the success in preparing a finely divided sol. It should show to the naked eye only traces of oil on the surface, even after standing many months, and appear transparent with a blue fluorescence. A "white"

appearance indicates that the oil particles are much larger than when the solutions are transparent and fluorescent. The material extracted by wet acetone from the olive oil is probably richer in free fatty acid than the original oil, and to this I have ascribed the fine emulsions obtained by this method. Adding the same oil drop by drop to 100 cc. acetone + 6 cc. water until no more dissolves even on prolonged shaking, I obtained from the solution coarse "white" emulsions on blowing 4 cc. of it into 100 cc. of water [Donnan, 1910; Dubrisay, 1913].

A freshly prepared oil sol does not differ in appearance from one many months old. It is found, however, that when an endeavour is made to prepare a flocculation diagram with some protein and an electrolyte solution the sol does not seem to be so sensitive to minute differences of protein or electrolyte concentration after long standing. And the sharp well defined arcs on the diagram give way to regions similar in shape but of blurred outline.

The *mastic sol* used in all these experiments was prepared from commercial gum mastic in the following manner. Excess of the gum was boiled under a reflux condenser with 99 per cent. alcohol for several hours, the vessel cooled and then allowed to stand for several days. The clear solution resulting contained 1·7 per cent. of total solids. Five cc. blown forcibly from a pipette into 95 cc. of conductivity water furnished an excellent sol, pale straw colour by transmitted light, and with a marked blue fluorescence. If the alcoholic solution is run out from the pipette into the water in the normal way a milky fluid results, the particles of the disperse phase of which are comparatively large. The difference between the solution resulting is in the two cases very striking; much more so than with oil or any other suspension I have observed.

Unlike the particles of the oil sol the mastic particles increased in size hourly in a very marked manner, though even after months of standing no flocculation occurred. Fresh suspensions were therefore prepared for each experiment. As their magnitude increased the particles seemed to change in size at a progressively lower rate.

Contrary to expectation it was found quite possible to measure hydrogen ion concentrations with accuracy in solutions containing mastic or oil in the colloidal condition.

The mastic and oil sols used contained small quantities of acetone and alcohol respectively. It was thought that possibly 1·7 per cent. of alcohol or acetone might affect the charge on the protein particles in the mixtures made, but no evidence of this could be observed. Samples of sols were

distilled until quite free from alcohol or acetone under diminished pressure, and then half made up to the original volume with the correct proportion of alcohol, and half made up with water. No difference in the behaviour of these two sols on flocculating with traces of gelatin and acid could be observed either in the rapidity or the limits of flocculation. Scarcely any difference was observable between the original sol and the same sol after distillation and making up to the original volume with the correct proportion of alcohol. In transport experiments the addition of 1·7 per cent. of alcohol to both middle and outer limbs made no difference to the direction of migration of gelatin in the neighbourhood of the isoelectric point.

The *gold sols* (Au_F) were prepared by Zsigmondy's [1901] formalin method followed by dialysis. The removal of the alkali rendered these sols less stable, and after several days' dialysis an increase in size of the particles was invariably observed. The finer sols (Au_P) obtained by the ethereal phosphorus method were also prepared.

Gelatin. Coignet Fils et Cie's Gold Label Gelatin was soaked in frequent changes of conductivity water in a hard glass vessel for several days till twelve hours' contact at 0° did not change 3 gemmho water to more than 5 gemmhos. No signs of mould or bacterial growth were observed. A solution, approximately 1 per cent., was prepared by dissolving in boiling distilled water and the exact strength determined by refractive index at 17·5°. Suitable dilution gave a concentration 10^{-3} from which other solutions could be made. A typical 10^{-3} gelatin solution had conductivity 9·6 gemmhos.

The effect of the dialysis could be demonstrated by comparing different concentrations of dialysed gelatin with corresponding concentrations of undialysed gelatin in their effect on mastic sol and N/10 sodium acetate. Equal volumes of gelatin solution, mastic sol and N/10 sodium acetate solution are taken in each test tube. Flocculation is more rapid and complete in the latter case and extends over a wider range of gelatin concentration—a result attributable to the additional electrolytes present.

Apparatus. Determinations of $H^{\cdot}$ ion concentration were in all cases made electrometrically, using a gas electrode of convenient type in conjunction with Michaelis' saturated KCl calomel electrode. The connecting fluid was saturated KCl and no correction for contact potential of the fluid investigated and saturated KCl was made. For nearly all of the determinations an electrode vessel of borosilicate glass with the electrode mounted in the same material and a ground joint [Walpole, 1914] was used.

Conductivity determinations were made by the Kohlrausch method, at

18°, in stoppered vessels of the Henry-Zörkendörfer pattern, using well-blacked electrodes.

As far as possible all solutions were stored in special bottles of hard glass. The test tubes employed were remarkable for their insolubility in water. As the interactions observed were many of them almost instantaneous, the slow absorption of carbon dioxide from the atmosphere by the mixture could be ignored.

The *ultramicroscopic* apparatus was Leitz' 1913 pattern illuminated by 5 amp. arc light. Observations on direction of migration in the electric field could be made in solutions containing only small quantities of electrolytes by using a cell 0·3 mm. deep provided with platinum electrodes [Chick and Martin, 1912].

Migrations in the electric field were also observed in an apparatus similar to that employed by Michaelis. Every due precaution was taken to avoid the fallacies contingent to the method.

In connection with the experimental part of this work I wish to express my indebtedness to my assistants Messrs R. Defries and S. Scott.

Summary.

A method is given by which the relationship of the phenomena of the inhibition or actuation of the aggregation of mixtures of sols and electrolytes, especially in the presence of proteins, may be coordinated diagrammatically on a quantitative basis.

This method has been applied to oil and mastic sols to which gelatin and hydrochloric acid have been added and the effect of the addition of sodium chloride and sodium acetate to the mastic-gelatin-hydrochloric acid mixtures has been studied in a similar manner.

The chief phenomena observed may be summarised here:

1. In the presence of not too much gelatin flocculation of mastic or oil sols may occur by the addition of a trace of acid quite unable to flocculate the sol alone. The same quantity of gelatin "protects" the sol against acid 6000 times more concentrated.

2. This aggregation may be observed equally well with gold sols and in all cases it is reversible.

3. In the absence of electrolyte except HCl, two critical mixtures are observed which are on the verge of flocculation. These have been worked out in the case of one particular mastic sol and called the "1st" and "2nd" critical mixtures.

1st critical mixture. The hydrogen ion concentration of this mixture is in the neighbourhood of the isoelectric point of gelatin but is probably on the alkaline side of this point. The particles are negatively charged in this mixture, and in contiguous mixtures whether flocculated or non-flocculated. Mixtures differing from this by having more gelatin, more acid or less acid do not flocculate.

2nd critical mixture. The hydrogen ion concentration of this mixture is $P_H^+ = 2{\cdot}9$. Mixtures having more acid or less acid flocculate, but here again more gelatin inhibits flocculation. The particles are positively charged in this mixture and in contiguous mixtures whether flocculated or non-flocculated.

4. In the presence of hundredth normal sodium chloride or sodium acetate there are also two critical mixtures showing precisely similar flocculation changes with changing acid or gelatin concentration. The following differences are noticeable:

(*a*) The two critical mixtures approximate much more closely than before in gelatin concentration.

(*b*) The *1st critical mixture* is either at or only just on the alkaline side of the isoelectric point of gelatin. The gelatin coated mastic particles are almost exactly electrically neutral. The *2nd critical mixture* in the presence of added electrolyte resembles the *2nd critical mixture* in the absence of added electrolyte in all respects including $H^{\cdot}$ ion concentration except that four times as much gelatin is present in the former case. Apparently the additional thickness of gelatin coat corresponding to the greater gelatin concentration counterbalances the flocculating effect of the added electrolyte.

5. Whether sodium chloride or acetate be present or no, the *1st critical mixture* contains more gelatin than the *2nd critical mixture.*

6. In any series of oil-gelatin or mastic-gelatin mixtures containing minimal traces of electrolyte, and differing only in $H^{\cdot}$ ion concentration, those which are flocculated are not those which contain electrically neutral particles. This lack of coincidence of the "point of maximum flocculation" and the point where the particles have no "electric charge" was pointed out in an earlier paper: an endeavour has been made here to analyse this phenomenon further.

7. Miss H. Chick's observation that "electrolyte-free" globulin at the reaction of maximum flocculation is not electrically neutral has been confirmed.

8. The wide difference of gelatin concentration (100 times) between the *1st critical mixtures* with and without salt addition is related to an area

of aggregation over which this phenomenon is more or less reversible experimentally by acid, alkali, or sodium chloride. The reversal by alkali is much more readily observed than that by acid.

9. A possible physical similarity between the particles of a mastic gelatin mixture and those of a euglobulin suspension is suggested. Preliminary attempts to make a suspension strictly comparable with euglobulin from pseudoglobulin and a sol from the lipoid of euglobulin have been made, and work on this subject is being continued.

These simple systems were originally examined in the hope that the results would assist in the elucidation of the phenomena met with in the investigation of the acid agglutination of bacteria. The system in the case of a bacterial suspension is, however, more complicated and this paper can only be regarded as a contribution to the general subject of flocculation.

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XXV. ACTION OF PEPSIN AND TRYPSIN ON ONE ANOTHER.

(Preliminary Paper.)

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Hitherto only a small amount of work has been done in investigating the action of enzymes on one another, and many of the earlier results are inconclusive or contradictory. It was stated by Corvisart (quoted by Wróblewski) that pepsin and trypsin were mutually destructive. Kühne [1876], on the other hand, found that pepsin digested trypsin, but that the reverse did not hold good. A few years later Engesser [1880] however found that pepsin had practically no action on trypsin, digestion being continued for two hours. Further uncertainty was introduced by the work of later authors.

Langley [1881] tested the action of certain enzymes on one another, taking the precaution to determine the action of acid or alkali of the appropriate strength on the enzymes in question. He found that pepsin was destroyed considerably more rapidly by trypsin in an alkaline solution than by alkali of the same strength acting on the pepsin alone. Langley also found that trypsin lost much of its digestive power when kept for some time in 0·05 % hydrochloric acid before being allowed to act on fibrin in alkaline solution, but he does not appear to have done any experiments himself to show the action of pepsin on trypsin. As the result of a considerable number of experiments on different enzymes, Wróblewski, Bednarski and Wojczynski [1901] conclude that not only does trypsin increase the destructive effect of alkali on pepsin, but also that pepsin increases the destructive effect of acid on trypsin. While different observers have found considerable variations in the range of hydrogen or hydroxyl ion concentration compatible with digestive power on the part of pepsin or trypsin respectively, there seems little doubt that the former enzyme has no appreciable action in an alkaline medium, trypsin on the other hand being active only in presence of alkali. Consequently previous investigations of the action of these enzymes

on one another appear to have been confined to discovering if pepsin would digest trypsin in acid solution, or if trypsin on the other hand would have a destructive action on pepsin in an alkaline medium.

In the light of the older view that enzymes were of a protein nature it was not unreasonable to expect that in such cases the pepsin for example would in an acid medium break up the trypsin into a number of less complex substances, and that thus the specific digestive properties of the trypsin would be lost.

Now that considerable doubt has been thrown on the protein nature of the enzyme molecule, however, such a breakdown of the molecule is by no means certain to take place in every case.

Within the last few years the mode of action of enzymes has been rendered clearer by experiments which appear to show that the enzyme acts on its appropriate substrate by means of different chemical groups or side chains.

Pollak [1905] found that on diluting a trypsin solution with a quantity of the same trypsin solution which had been previously heated to 70° much less digestion took place than in the case where the enzyme was diluted with a corresponding amount of water. Similar results were obtained by Schwartz [1905] in the case of pepsin.

Bayliss [1904] found that trypsin became altered in the above way on being heated, and suggested that the altered enzyme or "zymoid" still retained the power of combining with the substrate, but that the specific digestive power had been destroyed.

Bearn and Cramer [1907] also found that pepsin and rennin when heated to about 60° inhibited the action of the fresh enzyme when excess of the heated enzyme was added. Generally it was found that if the enzyme were heated to 100° this inhibitory power was lost, the reason probably being that the groups which combine with the substrate are themselves destroyed at 100°. Different enzyme preparations, however, varied considerably in their resistance to heat.

Although trypsin is a more energetic proteolytic enzyme than pepsin, nevertheless they are both capable of acting on a large number of proteins with the formation of similar hydrolytic products to a certain stage of digestion. This led to the idea that both enzymes might attack proteins by combining with the same side chains, the specific digestive action of the enzymes, however, being due to dissimilar side chains, the latter in the case of pepsin acting only in acid solution, and in the case of trypsin only in presence of alkali.

If this were the case we should expect that an excess of trypsin would inhibit the action of pepsin in acid solution, and similarly excess of pepsin would inhibit the action of trypsin in alkaline solution. The enzyme present in excess in such cases would thus act in a manner analogous to the zymoid of the other enzyme.

Experimental methods.

Various enzyme preparations were used in these experiments, such as Benger's Liquor Pepticus and Liquor Pancreaticus, and the pepsin and trypsin in scales or powder of Merck, Fairchild and Grübler.

The enzyme solutions were put in small flasks and a definite amount of dilute hydrochloric acid or sodium carbonate added. In each flask was then put an equal amount of the protein to be digested and the flasks were kept in a water bath at 37° for a certain time. Any slight accidental variations in temperature would affect all the flasks equally, and thus introduce no difficulty.

Two methods of estimating the amount of digestion were employed. The first, principally used for determining the amount of digestion by pepsin, was that of making the mixture of enzymes act on fibrin for a definite time. The undissolved fibrin was then filtered off and the nitrogen in a portion of the filtrate determined by Kjeldahl's method.

Since the object of these experiments was to determine the activity of one enzyme under different conditions, for example the relative digestive power of pepsin in dilute acid and of pepsin in the same strength of acid containing trypsin in addition, such a method of estimating the amount of digestion seems quite satisfactory, as the stages of digestion must presumably be the same in both cases, and where digestion has gone on for a definite time the amount of dissolved nitrogen must give a true indication of the digestive power of the enzyme under these conditions. The fibrin was always obtained from ox blood, and was minced, thoroughly washed till free from blood, suspended in water and heated to 70° in order to destroy any enzymes present. The water was then pressed out and the fibrin preserved in glycerol. When the fibrin was required for use, the glycerol was washed out with water, the excess of water pressed out and equal weighed amounts of the fibrin taken for experiment.

Control experiments were also carried out with the same amount of fibrin and acid or alkali, but no enzyme. No appreciable amount of fibrin was found to be dissolved in any of the controls.

The amount of nitrogen present in the solutions of enzymes used was also

determined in every case, and that amount deducted from the total nitrogen in solution at the end of the experiment. The difference represented the nitrogen of the protein dissolved by the enzyme.

The other method of determining digestive power employed in these experiments was that described by Hedin [1903]. The enzyme is allowed to act on a solution of caseinogen, and at the end of the period of digestion excess of tannic acid is added. The unchanged protein and proteoses are precipitated, and the nitrogen left in solution is estimated in the filtrate by Kjeldahl's method. This gives a measure of the amount of protein digested to the stage of peptone or amino-acids.

Control solutions of the enzymes and also of the caseinogen used were also treated in the same way with tannic acid, and the nitrogen in the filtrate allowed for in calculating the amount of protein digested. Toluene was added to each flask in order to prevent the action of bacteria. As a rule it was found that the fibrin method was the more suitable when digestion by pepsin was being studied, as pepsin digests caseinogen only very slowly to the stage at which it is not precipitated by tannic acid.

On the other hand, the caseinogen method was found more suitable for digestion by trypsin, the latter enzyme dissolving heated ox fibrin too slowly for convenience in experiment.

EFFECT OF EXCESS OF TRYPSIN ON THE DIGESTIVE POWER OF PEPSIN.

		Digestion in cc. of N/10 nitrogen
1.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl... ...	11·4
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	26·9
	1·4 g. fibrin added. Digestion 2·5 hours.	
2.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl... ..	9·0
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	26·3
	1·5 g. fibrin. Digestion 1·5 hours.	
3.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl... ...	9·9
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	28·8
	1·6 g. fibrin. Digestion 2·25 hours.	
4.	1 cc. pepsin, 10 cc. trypsin, 20 cc. water, 30 cc. N/10 HCl	37·1
	1 cc. pepsin, 20 cc. trypsin, 10 cc. water, 30 cc. N/10 HCl	14·2
	1 cc. pepsin, 30 cc. trypsin, 30 cc. N/10 HCl... ...	5·8
	1 cc. pepsin, 30 cc. water, 30 cc. N/10 HCl	47·6
	2 g. fibrin. Digestion 2·75 hours.	
5.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl.. ...	5·2
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	18·9
	1·7 g. fibrin. Digestion 1·25 hours.	
6.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl... ...	4·8
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	13·3
	1 g. fibrin. Digestion 2 hours.	

		Digestion in cc. of N/10 nitrogen
7.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl... ...	8·9
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	22·7
	1·1 g. fibrin. Digestion 1·5 hours.	
8.	1 cc. pepsin, 10 cc. trypsin, 20 cc. water, 30 cc. N/10 HCl	7·2
	1 cc. pepsin, 20 cc. trypsin, 10 cc. water, 30 cc. N/10 HCl	5·1
	1 cc. pepsin, 30 cc. trypsin, 30 cc. N/10 HCl... ...	4·2
	1 cc. pepsin, 30 cc. water, 30 cc. N/10 HCl	18·5
	1·8 g. fibrin. Digestion 1·5 hours.	

Of these experiments Nos. 1 to 4 were carried out with Benger's preparations, the others being with Merck's pepsin and trypsin (1 % solutions). As Liquor Pancreaticus is slightly acid, it was first neutralised, and a corresponding small amount of sodium chloride added to the control flask though this was probably not at all necessary.

It may be mentioned that exactly similar results were obtained by using Grübler's pepsin and trypsin in 1 % solution, and that many other experiments carried out with Benger's and Merck's preparations confirmed in every respect those described above, but those quoted are sufficient to show very clearly the effect of an excess of trypsin in inhibiting the digestive action of pepsin solutions. Experiments 4 and 8 also show that this inhibition varies with the proportion of trypsin present. If the amount of the latter enzyme be very large, the action of the pepsin is almost stopped entirely. This is what was to be expected if we assume that trypsin can combine with protein in acid solution.

The objection might be raised that the action of the trypsin was really due to the presence of protein or the products of protein digestion in the trypsin solutions used. That such is not the case, however, is shown by the following experiments.

		Digestion in cc. of N/10 nitrogen
9.	1 cc. pepsin, 20 cc. 2·5 % albumin, 30 cc. N/10 HCl ...	26·3
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	25·4
	1 g. fibrin. Digestion 1·5 hours.	
10.	1 cc. pepsin, 20 cc. 2 % caseinogen, 20 cc. water, 10 cc. N/10 HCl	29·7
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	24·9
	1 g. fibrin. Digestion 2 hours.	
	(The caseinogen was dissolved in N/10 HCl.)	
11.	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	31·9
	1 cc. pepsin, 20 cc. water, 30 cc. caseinogen	30·7
	1 cc. pepsin, 20 cc. water, 30 cc. albumin	37·6
	(The caseinogen and albumin were 1·5 % in N/10 HCl.)	
	1·2 g. fibrin. Digestion 1 hour.	

In these three experiments Liquor Pepticus was used.

		Digestion in cc. of N/10 nitrogen
12.	1 cc. pepsin, 20 cc. water, 20 cc. N/10 HCl	25·8
	1 cc. pepsin, 20 cc. water, 20 cc. caseinogen	24·4
	1 cc. pepsin, 20 cc. water, 20 cc. albumin	26·1
	1·1 g. fibrin. Digestion 1·75 hours.	
	(The caseinogen and albumin were 1 % in N/10 HCl.)	
	Merck's pepsin (0·1 %) was used in this experiment.	
13.	1 cc. pepsin, 20 cc. water, 20 cc. N/10 HCl	32·3
	1 cc. pepsin, 20 cc. water, 20 cc. caseinogen	32·6
	1 cc. pepsin, 20 cc. water, 20 cc. albumin	31·7
	1·2 g. fibrin. Digestion 0·75 hour.	
	(Caseinogen and albumin 1 % in N/10 HCl.)	
	Grübler's pepsin (1 %) was used in this experiment.	

It is clear from these experiments that digestion of a solid protein such as fibrin is not less rapid when there is also present in solution a protein such as caseinogen or egg albumin. The action of the pepsin on the fibrin seems to begin immediately in such a case, there being no interval during which the protein in solution is first broken down into less complex substances.

The action of the trypsin is not an actual destruction of the pepsin. This is at once seen if the amounts of fibrin used vary considerably. It has been shown by Hedin [1905] that the amount of caseinogen digested in a given time by a certain amount of trypsin increases with the concentration of the substrate, until a maximum is reached when sufficient substrate is present to unite with the whole of the trypsin.

Similar results are obtained in the digestion of fibrin by pepsin.

		Digestion in cc. of N/10 nitrogen
14.	1 cc. pepsin, 20 cc. trypsin, 20 cc. N/10 HCl, 0·9 g. fibrin	9·9
	1 cc. pepsin, 20 cc. trypsin, 20 cc. N/10 HCl, 2·7 g. fibrin	28·8
	1 cc. pepsin, 20 cc. water, 20 cc. N/10 HCl, 0·9 g. fibrin...	20·7
	1 cc. pepsin, 20 cc. water, 20 cc. N/10 HCl, 2·7 g. fibrin...	65·6
	Digestion 1 hour.	
15.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 0·9 g. fibrin	10·9
	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 3·4 g. fibrin	42·2
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl, 0·9 g. fibrin...	25·5
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl, 3·4 g. fibrin...	94·1
	Digestion 0·75 hour.	
	In these two experiments Benger's preparations were used.	
16.	0·8 cc. pepsin, 25 cc. trypsin, 30 cc. N/10 HCl, 1 g. fibrin	7·9
	(*a*) 0·8 cc. pepsin, 25 cc. trypsin, 30 cc. N/10 HCl, 3·5 g. fibrin	17·1
	(*b*) 0·8 cc. pepsin, 25 cc. water, 30 cc. N/10 HCl, 1 g. fibrin	22·4
	0·8 cc. pepsin, 25 cc. water, 30 cc. N/10 HCl, 3·5 g. fibrin	84·5
	Digestion 0·75 hour.	
17.	0·8 cc. pepsin, 25 cc. trypsin, 30 cc. N/10 HCl, 0·7 g. fibrin	4·9
	(*a*) 0·8 cc. pepsin, 25 cc. trypsin, 30 cc. N/10 HCl, 5 g. fibrin	12·7
	(*b*) 0·8 cc. pepsin, 25 cc. water, 30 cc. N/10 HCl, 0·7 g. fibrin	18·5
	0·8 cc. pepsin, 25 cc. water, 30 cc. N/10 HCl, 5 g. fibrin...	121·9
	Digestion 0·75 hour.	

		Digestion in cc. of N/10 nitrogen
18.	0·8 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 0·5 g. fibrin	4·7
	0·8 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 1·5 g. fibrin	12·6
	0·8 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 7 g. fibrin	13·1
	0·8 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl, 0·5 g. fibrin	10·5
	Digestion 0·5 hour.	

0·5 % pepsin and 1 % trypsin (Grübler) were used in these three experiments.

Those experiments show well the effect of varying the quantity of fibrin added to the enzyme solutions. It is clear that the effect of adding an excess of trypsin to a given amount of pepsin can be apparently counteracted by increasing the amount of fibrin. The reason evidently is that when the fibrin is increased in amount a larger number of pepsin molecules can carry on their digestive action, although in those experiments at least the amount of digestion by pepsin + trypsin relative to that by pepsin alone is no greater when much fibrin is present than when a smaller amount of the substrate only is used.

In experiments 16 and 17 it will be noticed that the amount of digestion in (*a*) is still less than that in (*b*) in spite of the fact that the amount of fibrin in the former is considerably greater than in the latter case. In these two experiments the proportion of trypsin to pepsin is probably higher than in experiments 14 and 15, consequently the proportion of fibrin should have been correspondingly higher in (*a*) than in (*b*) to produce the same effect. By using still smaller amounts of fibrin as in No. 18 this difficulty is got over, and in this last experiment we see again that by employing suitable proportions of fibrin it is possible to get a greater amount of digestion by the pepsin + trypsin than by the pepsin alone.

Since it has been clearly shown that the inhibition of pepsin by excess of trypsin is not due to the presence of soluble protein in the trypsin solution, also that it is not due to the pepsin being destroyed by the trypsin, it seems reasonable to suppose that this inhibition is due to the trypsin itself. If this were the case, we should expect that such an inhibition would no longer occur if the trypsin were destroyed.

The simplest way appeared to be by boiling the solution of trypsin before adding it to the pepsin. This, however, proved somewhat ineffective as the following show.

		Digestion in cc. of N/10 nitrogen
19.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl	9·9
	1 cc. pepsin, 20 cc. boiled trypsin, 30 cc. N/10 HCl ...	16·8
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	28·8
	1·6 g. fibrin. Digestion 2·25 hours.	
20.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl	15·4
	1 cc. pepsin, 20 cc. boiled trypsin, 30 cc. N/10 HCl ...	20·7
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	40·2
	1·7 g. fibrin. Digestion 1·5 hours.	

In these two experiments Liquor Pepticus and Liquor Pancreaticus were used. The latter was boiled under a reflux condenser for about 30 minutes and filtered. The precipitate was found to be entirely and readily soluble in the same concentration of pepsin as was used for the main experiment. As mentioned in a previous paper by me [1914] this trypsin preparation is slightly acid and retains a considerable proportion of its digestive power even after being boiled. In the last two experiments therefore, the digestive power of the trypsin was probably not entirely destroyed, or at least the power of the trypsin to unite with its substrate. In this case it is easy to understand why boiled trypsin still inhibits the action of pepsin on fibrin.

It may be mentioned here that it was through these unexpected results that I was led to investigate the effect of heat on trypsin more fully. As it has been shown in the paper referred to and by other workers that trypsin loses its digestive power readily when boiled in neutral or alkaline solution, I tried the effect of trypsin, which had been thus boiled, on pepsin, but found that it was still effective in inhibiting the digestion of fibrin by pepsin.

There is reason to believe, from preliminary experiments, that it is much harder completely to inactivate the trypsin molecule than the pepsin molecule, so that the zymoid modification of trypsin would still inhibit the action of pepsin even after prolonged heating of the trypsin solution. Different trypsin preparations also are found to behave differently in this respect. It was also found by Bearn and Cramer that different preparations of pepsin varied considerably in their behaviour to heat. The results with trypsin will be described in a later paper on this subject.

Trypsin was found to inhibit the action of pepsin on caseinogen solutions also, but the effect of pepsin on this substrate is slight, so far, at least, as rendering the nitrogen non-precipitable by tannic acid goes.

		Digestion in cc. of N/10 nitrogen not ppted. by tannic acid
21.	1 cc. pepsin, 20 cc. trypsin, 15 cc. N/10 HCl... ...	3·1
	1 cc. pepsin, 20 cc. water, 15 cc. N/10 HCl	6·2
	20 cc. of 1 % caseinogen in N/20 HCl added. Digestion 4 hours.	
22.	1 cc. pepsin, 20 cc. trypsin, 30 cc. 2 % caseinogen in N/10 HCl	9·8
	1 cc. pepsin, 20 cc. water, 30 cc. 2 % caseinogen in N/10 HCl	14·3

Liquor Pepticus and Liquor Pancreaticus used in these two experiments.

In No. 22, digestion lasted 18 hours, so that while these experiments show that trypsin again inhibits pepsin, they show still more clearly that this method is unsuitable for estimating the digestive activity of pepsin solutions.

EFFECT OF EXCESS OF PEPSIN ON THE DIGESTIVE ACTION OF TRYPSIN.

The experiments were carried out similarly to those already described. When Liquor Pepticus was used as the pepsin solution, a considerable allowance had to be made for the acidity of this solution, the control solution having sodium chloride added in amount corresponding to that in the neutralised pepsin solution.

		Digestion in cc. of N/10 nitrogen
23.	1 cc. trypsin, 20 cc. pepsin, 40 cc. 1 % Na_2CO_3 ...	6·7
	1 cc. trypsin, 20 cc. water, 40 cc. 1 % Na_2CO_3 ...	12·2
	1 g. fibrin. Digestion 2 hours.	
24.	1 cc. trypsin, 20 cc. pepsin, 40 cc. 1 % Na_2CO_3 ...	7·9
	1 cc. trypsin, 20 cc. water, 40 cc. 1 % Na_2CO_3 ...	18·6
	1·2 g. fibrin. Digestion 2 hours.	
25.	1 cc. trypsin, 10 cc. pepsin, 20 cc. water, 35 cc. 1 % Na_2CO_3	26·4
	1 cc. trypsin, 20 cc. pepsin, 10 cc. water, 35 cc. 1 % Na_2CO_3	21·1
	1 cc. trypsin, 30 cc. pepsin, 35 cc. 1 % Na_2CO_3 ...	12·8
	1 cc. trypsin, 30 cc. water, 35 cc. 1 % Na_2CO_3 ...	33·3
	2 g. fibrin. Digestion 1·5 hours.	

Liquor Pancreaticus and Liquor Pepticus were used in these experiments.

It will be seen from the above that an excess of pepsin inhibits the action of trypsin on fibrin, the amount of inhibition varying with the amount of pepsin added.

On the whole it was found that much more digestion was effected when caseinogen was used as the substrate, and the nitrogen estimated in the filtrate from the tannic acid precipitate.

		Digestion in cc. of N/10 nitrogen not ppted. by tannic acid
26.	1 cc. trypsin, 20 cc. pepsin, 40 cc. caseinogen ...	20·3
	1 cc. trypsin, 20 cc. water, 40 cc. caseinogen ...	46·9
	Caseinogen was 2 % in 1 % Na_2CO_3. Digestion 3 hours.	
27.	1 cc. trypsin, 20 cc. pepsin, 40 cc. caseinogen ...	28·2
	1 cc. trypsin, 20 cc. water, 40 cc. caseinogen ...	53·6
	Caseinogen as above. Digestion 4 hours.	

The effect of a soluble protein such as caseinogen on the digestion of fibrin by trypsin was tested by two experiments.

		Digestion in cc. of N/10 nitrogen
28.	1 cc. trypsin, 20 cc. caseinogen, 20 cc. N/10 Na_2CO_3 ...	19·3
	1 cc. trypsin, 40 cc. N/10 Na_2CO_3	16·3
	1 g. fibrin. Digestion 2 hours.	
	The caseinogen was 2 % in N/10 Na_2CO_3.	
29.	1 cc. trypsin, 20 cc. caseinogen, 20 cc. N/10 Na_2CO_3 ...	17·9
	1 cc. trypsin, 40 cc. N/10 Na_2CO_3	16·8
	0·8 fibrin. Digestion 2 hours.	
	The caseinogen was 2 % in N/10 Na_2CO_3.	

These two experiments show that the presence of excess of a soluble protein such as caseinogen does not delay the digestion of fibrin by trypsin. Egg albumin, as is well known, inhibits the action of trypsin, and I found that only a small amount of fibrin was digested if albumin was added in place of the caseinogen in these two experiments. The above results show, however, that the inhibition of trypsin by excess of pepsin is not due to the trypsin digesting the proteins in solution before attacking the fibrin.

Several experiments were carried out to see if pepsin solutions which had been boiled lost their power of inhibiting the action of trypsin. Here again, as in the corresponding experiments with boiled trypsin in excess acting on pepsin, the results varied considerably.

		Digestion in cc. of N/10 nitrogen not ppted. by tannic acid
30.	1 cc. trypsin, 20 cc. pepsin, 40 cc. caseinogen ...	14·9
	1 cc. trypsin, 20 cc. boiled pepsin, 40 cc. caseinogen ...	40·3
	1 cc. trypsin, 20 cc. water, 40 cc. caseinogen ...	52·8

The caseinogen was 2 % in N/10 Na_2CO_3. Digestion 3 hours.

Liquor Pepticus and Liquor Pancreaticus were used in this experiment.

This shows that pepsin by being boiled loses most of its power to inhibit trypsin. In this case the pepsin had been boiled for about 10 minutes. Probably it still retained some of its zymoid properties, which would have been destroyed by more prolonged heating.

Although I had previously noticed that boiled trypsin still inhibited the action of pepsin, it was in the course of this last experiment that I found, in one of the controls containing boiled trypsin and caseinogen, that the boiled enzyme had brought about a considerable amount of digestion of the protein. I was led from that to consider the question of the resistance of trypsin to heat, and since then have been unable to get clear evidence of the destruction of the inhibitory power of pepsin by heat. As was shown by Bearn and Cramer, different preparations of pepsin vary widely in their resistance to heat, and it may be that in my experiments the solutions have always retained their power of combining with the substrate. This question is being now investigated, and the results will be published in a later paper.

It may be mentioned that Bayliss found that he could not always produce the zymoid modification of trypsin, or at least demonstrate the action of the zymoid, and it is evident from the experiments described in this preliminary paper that a number of points have still to be settled in regard to the varying behaviour of different enzyme solutions after being heated.

SUMMARY.

Excess of trypsin inhibits the digestive action of pepsin in acid solution. Such inhibition is not due simply to the presence of protein associated with the trypsin. Nor is it due to the pepsin being destroyed by the trypsin.

The power of trypsin to inhibit pepsin is to some extent lost if the trypsin solution is boiled before being added to the pepsin.

It is suggested that trypsin can combine with protein in acid solution, so that although unable to digest such protein, an excess of trypsin would prevent pepsin from combining fully with the protein.

Similarly an excess of pepsin inhibits the digestive action of trypsin in alkaline solution. The amount of inhibition in this case is usually less than that caused by an excess of trypsin on the digestive power of pepsin in acid solution.

This inhibition again is not simply due to the presence of protein associated with the pepsin. Heating the pepsin solution destroys to a considerable extent its power to inhibit trypsin.

In this case it is suggested that the pepsin acts by combining with the protein in alkaline solution, thus preventing the digestion of the protein by the trypsin.

In both cases the amount of inhibition produced depends on the relative proportions of the enzymes present. Thus, the greater the amount of pepsin, the less digestion will be effected by a given amount of trypsin acting in alkaline solution.

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XXVI. THE FLOWER PIGMENTS OF *ANTIRRHINUM MAJUS*. III. THE RED AND MAGENTA PIGMENTS.

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An account of the Mendelian factors for flower colour in *Antirrhinum*, of the preparation of the pigment and of the analyses of the yellow pigments has been given in previous communications [Wheldale, 1913; Wheldale and Bassett, 1913, 1914].

Of the yellow pigments in *Antirrhinum*, only two occur in quantity and are concerned in the inheritance of colour. One is present in the so-called ivory variety and has been identified with the flavone, apigenin, $C_{15}H_{10}O_5$; the other is present (in addition to apigenin) in the yellow variety and has been identified with the flavone, luteolin, $C_{15}H_{10}O_6$. The ivory variety is dominant to the yellow and contains a factor "I" which is absent from yellow and may be expressed as the power to inhibit the formation of luteolin. The true white or albino variety does not contain either apigenin or luteolin.

The remaining varieties contain anthocyanin in addition to flavones and may be enumerated as follows:

1. Yellow tinged with bronze.
2. Ivory tinged with rose doré.
3. Bronze.
4. Rose doré.
5. Yellow tinged with crimson.
6. Ivory tinged with magenta.
7. Crimson.
8. Magenta.

It has hitherto seemed probable that, in addition to flavones, one anthocyanin pigment only—red—is responsible for varieties 1–4, rose doré being coloured by red anthocyanin and apigenin, and bronze by red anthocyanin and luteolin. This assumption has now been confirmed and also the similar view that varieties 5–8 contain only magenta anthocyanin; crimson being coloured by magenta anthocyanin and luteolin, and magenta by the same anthocyanin and apigenin.

The purification of red and magenta anthocyanins was carried out as follows:—The crude pigment (obtained by hydrolysis of the glucosides from flowers containing, of course, flavone in addition to anthocyanin) is finely powdered and extracted with absolute alcohol and in this way is purified from a considerable amount of brownish insoluble substance which is probably a decomposition product produced by boiling with sulphuric acid during the hydrolysis of the glucoside. The alcohol extract is taken to dryness again, finely ground, and extracted in a Soxhlet thimble suspended immediately above boiling ether. After some months most of the flavone pigments are removed by the ether, the process being continued with fresh quantities of ether till the latter remains practically colourless after boiling for a few days. The residue consisting almost entirely of anthocyanin, is then taken up in the minimum amount of absolute alcohol and a large volume of ether added which precipitates the anthocyanin, any flavone present remaining in solution. The precipitated anthocyanin is filtered off, dried, and again extracted with ether to remove traces of flavone.

Red anthocyanin thus prepared is an Indian red powder. We have not yet been able to obtain anthocyanin as a satisfactory crystalline product. It does not melt even when heated to 350°. It is readily soluble in absolute alcohol and pyridine, slightly soluble in dilute acids, ethyl acetate and acetone, almost insoluble in water and insoluble in ether, chloroform and benzene. In concentrated sulphuric acid it forms a reddish solution with a slight green fluorescence. It is soluble in alkalies to a green solution. With ferric chloride solution it gives a green colouration changing to brown. Lead acetate forms a brownish yellow precipitate, but barium, silver, cadmium and mercury do not form precipitates.

Pure magenta anthocyanin is a magenta red powder with similar properties and solubilities to the red. In concentrated sulphuric acid it gives a red solution with a slight greenish fluorescence. It is soluble to a green solution in alkalies. With ferric chloride solution it gives a green colouration changing to blue. With lead acetate it forms a dull green precipitate of a lead salt. In alkaline solutions it is strongly fluorescent, green by transmitted, red by reflected light.

The combustion results for pure red anthocyanin derived from different varieties were:

		C	H	O (by difference)
From rose doré		51·93 %	5·02 %	43·05 %
From bronze		51·37	5·05	43·58
,, ,, ...		52·12	4·97	42·91
	Mean	51·81	5·01	43·18

Of magenta anthocyanin from different varieties:

From magenta	50·26 %	4·89 %	44·85 %
From ivory tinged with magenta	50·68	5·54	43·78
From crimson	50·56	4·90	44·54
Mean	50·50	5·11	44·39

The corresponding numbers for the yellow pigments are as follows:

Apigenin	66·66 %	3·70 %	29·64 %
Luteolin...	62·90	3·49	33·61

The anthocyanins were always obtained in amorphous form, but although we were unable to get a crystalline product, the combustion results, combined with the failure to effect any separation even though a large number of solvents were used, made it appear certain that the pigments were single substances and not constant mixtures, a view confirmed by the fact that the same substance could be obtained from different varieties.

This note is suggested by the following observations. In working with the yellow pigments we, for a long time, obtained a mixture of apigenin and luteolin of constant melting point and great difficulty was experienced in separating the constituents, but in this case the mixture gave very variable combustion results, quite in contrast with those of the anthocyanins quoted above.

Attempts were made to determine the molecular weight by depression of freezing point, using phenol as a solvent, but the results, though consistent in a series of experiments with anthocyanin from different varieties, were obviously far too low. In the case of magenta, the average value was 150, of red 380. Since the value for magenta is clearly too small, it is probable that the red is also below the correct value. The remarkable agreement between the results in the series of determinations carried out suggests however that the numbers obtained bear a simple relation to the molecular weights and that the low values are to be explained by some type of dissociation in the phenol.

Acetic acid and various other solvents did not dissolve sufficient substance to give readable depressions.

Attempts to determine the molecular weight by elevation of boiling point in absolute alcohol gave mean values of 572 for red and 717 for magenta. The elevation of the boiling point, for the concentrations it was possible to use, was so slight that the possible experimental error might be very considerable. Added to this, there appeared to be some change in the anthocyanin during the experiment and towards the end varying amounts were precipitated.

The combustion results give as simplest formulae, $C_8H_9O_5$ for the red and $C_{15}H_{18}O_{10}$ for the magenta. The boiling point determination of the molecular weight would appear to indicate that the molecule is $3(C_8H_9O_5)$, i.e. $C_{24}H_{27}O_{15}$ which has a molecular weight of 555 for the red, and $2(C_{15}H_{18}O_{10})$, i.e. $C_{30}H_{36}O_{20}$ which has a molecular weight of 716 for the magenta.

Lead salts of both the anthocyanins were prepared by mixing alcoholic solutions of lead acetate and anthocyanin. The precipitates were washed with hot alcohol, dried at 130° C. and the lead determined as $PbSO_4$.

In the case of red, 1 gram-atomic weight of lead was found to be equivalent to 224 g. of anthocyanin. Taking the molecular weight of the anthocyanin to be 555, this would indicate that 5 atoms of lead combine with 2 molecules of the pigment and the molecular weight of red anthocyanin calculated in this way from the lead equivalent would be 560.

In the case of magenta, 1 gram-atomic weight of lead was found to be equivalent to 204 g. of anthocyanin. Taking the molecular weight of magenta anthocyanin to be 716, this would indicate that 7 atoms of lead combine with 2 molecules of the pigment and the molecular weight of magenta anthocyanin calculated from the lead equivalent would be 714.

An attempt was made to estimate the number of hydroxyl groups present in the anthocyanin molecule by means of Zerewitinoff's [1907] modification of Hibbert and Sudborough's method. This consists in dissolving the substance in thoroughly dried pyridine, treating it in a suitable apparatus with a considerable excess of methyl magnesium iodide dissolved in dry amyl ether, and collecting the gas evolved. Each hydroxyl group causes the evolution of a molecule of methane. It should be noted that "hydroxyl groups" as determined by this method include those forming part of carboxyl groups, and also such ketone groups as can give rise to hydroxyl by a tautomeric change.

The values obtained indicate that the red anthocyanin, assuming the formula to be $C_{24}H_{27}O_{15}$, contains twelve hydroxyl groups as defined above, while the magenta, taking the formula as $C_{30}H_{36}O_{20}$ contains fifteen hydroxyl groups.

It will be seen that in the formation of the lead salts, a number of the hydroxyl groups do not react.

As far as our researches are concerned, it still remains to be shown that anthocyanin is a flavone derivative. The order of magnitude indicated for the molecular weights suggests that the anthocyanins of *Antirrhinum*, if flavone derivatives, are considerably more complex than flavones and may be formed by condensation of two molecules accompanied by oxidation or by condensation of a flavone with phenols or hydroxybenzoic acids.

In a recent paper, Willstätter [1913] gives some account of the extraction and analyses of pigments (and their derivatives) from the Corn-flower. Three pigments are identified; a purple pigment which he considers to be a flavone derivative and which is an acid; a blue pigment, the potassium salt of the purple, and a red pigment which is a salt, of the oxonium type, of the anthocyanin with an acid present in the plant.

The relation between the magenta and red pigments of *Antirrhinum* cannot be of the nature of that suggested by Willstätter for the pigments of the Corn-flower. It seems that the anthocyanins of the two genera differ considerably from each other. For instance Willstätter's anthocyanins form oxonium salts with mineral acids with great ease, whereas we have entirely failed to obtain such salts by any method. The very dissimilar range of colour varieties thrown off in the two cases also lends support to the view that the anthocyanins concerned are different in character.

From the standpoint of genetics, the chief items of interest contained in the present paper are the following:

1. There are only two kinds of anthocyanin in *Antirrhinum*, red and magenta. Mixed with ivory pigment (apigenin) the colours of red and magenta are unaffected, but a mixture with yellow pigment (luteolin) gives two other colours, bronze and crimson respectively. Both red and magenta anthocyanin may also occur in varying amounts giving rise to tinged, pale and deep varieties.

2. Both red and magenta anthocyanin contain a considerably higher percentage of oxygen than the flavones, and of the two, the percentage in magenta is the higher. Hence if the anthocyanins are derived from flavones, the process must be in part one of oxidation.

3. There is evidence also that the anthocyanin molecules are larger than the flavone molecule. Hence, again, if a flavone constitutes the chromogen, condensation must take place, either of two flavone molecules, or of a flavone with one or more molecules of an aromatic acid or phenol, etc.

We were unfortunately obliged to discontinue work at this point owing to our supply of pure material being exhausted, but we hope to carry out further investigations and to try to elucidate the constitution of anthocyanins.

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XXVII. THE CONSTITUTION OF PSEUDO-MUSCARINE ("SYNTHETIC MUSCARINE").

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Pseudo-muscarine or "synthetic muscarine" as the base has generally been termed was first obtained by Schmiedeberg and Harnack [1877]. Schmiedeberg and Koppe [1869] had obtained from the "Fly Agaric" (*Amanita muscaria*) a very powerfully active base "muscarine," which in its chemical behaviour closely resembled choline with which it was found associated in the fungus. Harnack [1876] showed that the muscarine obtained by Schmiedeberg and Koppe was still mixed with choline, and effected a purification of the base by repeated recrystallisation of the aurichloride. From the analyses of this salt he assigned to muscarine the formula $C_5H_{15}O_3N$, and on account of the close chemical relationship of the base to choline and its presence in the fungus with the latter base, suggested that its constitution could be expressed by the formula

$$(OH)\,N\,(CH_3)_3CH_2\,.\,CH\,(OH)_2,$$

muscarine thus being represented as a hydrated aldehyde derived from choline.

This hypothesis was apparently confirmed a little later when Schmiedeberg and Harnack [1877], by evaporating to dryness a solution of choline (or still better the platinichloride of the base) in concentrated nitric acid, obtained a base which they stated was chemically and physiologically indistinguishable from the natural muscarine obtained from *Amanita muscaria*. This base was, therefore, regarded as "synthetic" muscarine, and the supposition that muscarine was indeed an oxidation product of choline was apparently confirmed.

This straightforward solution of the problem of the constitution of muscarine was however shown to be incorrect by Boehm [1885] who found that "synthetic" muscarine, while closely resembling the natural base in physiological action, in some respects differed markedly, the most important

differences being that the synthetic base showed a powerful curare-like action which the natural base does not possess, and that the synthetic base did not constrict the mammalian pupil whereas natural muscarine does.

The problem was still further complicated by the fact that the aldehyde corresponding to choline, the so-called "betaine aldehyde" which was first synthesised by Berlinerblau [1884] and later by E. Fischer [1893] (who proved its constitution by oxidising it to betaine), was found to differ considerably in its action from both the natural and "synthetic" muscarines.

The problem was later re-investigated by Nothnagel [1894]. He obtained both natural and "synthetic" muscarine; the former from *Amanita muscaria*, the latter by Schmiedeberg and Harnack's method from choline. He too stated that so far as could be judged from the small amount of material available, the natural muscarine was chemically identical with the synthetic base, and confirmed Schmiedeberg and Harnack's formula for the latter. He also repeated and confirmed Berlinerblau's work. The physiological action of these bases was examined by Hans Meyer who confirmed Boehm's observations with regard to synthetic and natural muscarine and in addition pointed out further differences in their action.

Briefly then it appeared that there were two chemically indistinguishable bases which possessed different physiological actions and for this no satisfactory explanation has as yet been put forward.

Recently during an investigation into the nature of a muscarine-like base which is present in extracts of ergot, and was found to be acetyl-choline, as already described [Ewins, 1914], I had occasion to prepare some pseudo-muscarine for purposes of comparison and it occurred to me that, in view of the facts detailed above, confirmation of the constitution of this base was desirable, and that further examination might throw some light on the general problem. The preparation of the base gave no difficulty and a pure crystalline platinichloride was obtained, the base from which showed all the characteristic physiological effects[1] of synthetic muscarine as described by Boehm [1885], Hans Meyer [v. Nothnagel, 1894] and Honda [1911].

The formula assigned by Schmiedeberg and Harnack to their platinichloride and confirmed by Nothnagel was as follows:

$$[Cl\,N\,(CH_3)_2CH_2\,.\,CH\,(OH)_2]_2\,PtCl_4\,.\,2H_2O.$$

The salt thus contained two molecules of water of crystallisation. It is to be noted, however, that these were not completely removed, except by heating to a temperature of 130–135°.

[1] The physiological experiments involved in this investigation were in all cases carried out by Dr H. H. Dale.

As a preliminary step it was decided to attempt to confirm the above formula. The platinichloride was obtained "air dry" by standing in an ordinary desiccator over calcium chloride. The salt then remained unaltered in weight when placed *in vacuo* in a desiccator over sulphuric acid, and when heated under ordinary pressure in a steam bath (98°). Loss of weight occurred when the salt was heated to a temperature of about 115°, but it was found that by heating to 130–135° the loss of weight was considerably greater than that required for the loss of two molecules of water, and it was evident that the salt was undergoing decomposition. Finally it was found that the air dry platinichloride could be heated *in vacuo* at 100° for some hours without undergoing any loss of weight; a fact which afforded conclusive evidence of the absence of water of crystallisation.

An examination of the analytical data recorded by Schmiedeberg and Harnack, as well as by Nothnagel, further showed that there was no record of a determination of the nitrogen content of the molecule, although an otherwise complete analysis had been carried out. The platinichloride was accordingly analysed for nitrogen according to Dumas' method, when figures were obtained showing the presence of four nitrogen atoms in the molecule, i.e. two atoms of nitrogen in each molecule of free base. Accepting the remaining analytical data as more or less accurate for the supposedly hydrated platinichloride the actual formula for this salt was presumably the following:

$$(C_5H_{13}O_2N_2)_2\,PtCl_6.$$

Subsequently complete analysis proved this to be correct.

For the constitution of this base the two most probable formulae were the following:

$$\underset{\text{I}}{(OH)\,N\,(CH_3)_3\,CH_2\,.\,CH_2\,.\,NO_2}, \qquad \underset{\text{II}}{(OH)\,N\,(CH_3)_3\,CH_2\,.\,CH_2\,.\,O\,.\,NO.}$$

Formula (I) is that of an aliphatic nitro-derivative, the nitrogen being directly linked to a carbon atom while (II) represents the base as the choline ester of nitrous acid. That formula (II) correctly represents the constitution of the base is shown by the following facts:

In the first place the base gives a typical Liebermann's nitroso-reaction with phenol and sulphuric acid, which would not be the case if it were a nitro derivative. In the second place, on hydrolysis of the base by means of dilute acid or alkali, nitrous acid is very readily liberated, and choline may be recovered from the solution.

This base was actually obtained and recognised as such by Nothnagel

[1894] (who termed it "nitroso-choline") in the preparation of pseudo-muscarine by Schmiedeberg and Harnack's method. He considered this substance, however, to be a bye-product of the reaction, chiefly on account of a difference of crystalline form of the platinichlorides of the base and of pseudo-muscarine respectively. It was, however, remarked by Schmiedeberg and Harnack [1877] that the platinichloride of pseudo-muscarine (synthetic muscarine) which crystallises ordinarily in octahedra might also form "Federfahne ähnliche Aggregaten," a description which corresponds with that of "federbartartige Krystallen" recorded by Nothnagel for the platinichloride of "nitroso-choline."

That the supposed difference of constitution of these two bases in reality had very little foundation is supported by the analytical figures recorded by Nothnagel [1894] and shown in the following table:

Pseudo-muscarine platinichloride $[N(CH_3)_3CH_2.CH(OH)_2]_2 PtCl_6 2H_2O$ Found	Nitroso-choline platinichloride $[N(CH_3)_3CH_2CH_2O.NO]_2 PtCl_6 2H_2O$ Found	Choline nitrous ester platinichloride (anhydrous) Calculated
C = 17·66	17·69	17·8
H = 4·92	3·70	3·85
N —	8·31	8·31
Cl = 31·07	—	31·6
Pt = 28·54, 28·60, 28·44	28·73	28·93
Aurichloride	Aurichloride	Aurichloride
Au = 42·66	Au = 41·79	Au = 41·7

It will be observed that Nothnagel also formulates the "nitroso-choline" platinichloride as containing two molecules of water of crystallisation which, he states, were not removed by long heating at 100°. Nevertheless after drying the salt at 100° he obtained figures which, as is seen, agree well for the anhydrous salt. This fact was also pointed out by Schmidt and Wagner [1904], who obtained "nitroso-choline" by the prolonged action of concentrated nitric acid on choline at the ordinary temperature. The identity of this base with pseudo-(synthetic) muscarine is further supported by the similarity of the melting points of the aurichlorides ("pseudo-muscarine" aurichloride m.p. 234° after sintering from 174°; "nitroso-choline" aurichloride m.p. 240°) and by the fact stated by Nothnagel that the solubilities of the platinichloride are very similar.

In view, therefore, of the foregoing facts, it must be accepted that by the action of concentrated nitric acid upon choline as originally described by Schmiedeberg and Harnack, only one base yielding a platinichloride comparatively little soluble in cold water is produced. This base, which, as will

be shown in detail elsewhere by Dale, produces all the physiological effects described by various authors as brought about by pseudo-muscarine (synthetic muscarine), is, in fact, the choline ester of nitrous acid, and the constitution originally suggested for this base by Schmiedeberg and Harnack must be considered to be disproved.

If the formula ascribed by Harnack [1876] to the natural base be accepted as correct the difference in physiological action of the two bases is easily accounted for by their different chemical constitutions. It is, however, by no means certain that this formula can be accepted as correctly representing the constitution of the base. The facts that many choline-esters have a high degree of activity [Hunt and Taveau, 1911], which in the case of acetylcholine has been shown to approximate to the muscarine type of action [Dale, 1914], and that the so-called pseudo-muscarine is the choline-ester of nitrous acid, make it possible that muscarine itself may be a base of this class. In such a case recrystallisation of the aurichloride might well be accompanied by partial hydrolysis of the base.

With these possibilities in mind I prepared the choline-ester of nitric acid $(OH)\,N\,(CH_3)_3\,CH_2\,.\,CH_2\,.\,O\,.\,NO_2$ by the method described by Schmidt and Wagner [1904]. This base, again, is possessed of very considerable activity and in fact its action in some respects resembles that of natural muscarine much more closely than does that of pseudo-muscarine; nevertheless it still produces a marked curare effect upon frogs. How far these facts have any bearing upon the question of the constitution of natural muscarine must, however, for the present remain an open question.

Experimental.

Preparation of pseudo-muscarine. 3·8 grams of pure choline platinichloride were dissolved in about 5 cc. of concentrated nitric (D 1·4) on the water bath and then rapidly evaporated just to dryness on a sand bath. The reaction product was washed successively with absolute alcohol and once or twice with small quantities of cold water to remove the bulk of the unchanged choline platinichloride. The residue was crystallised from a little hot water. The platinichloride of pseudo-muscarine separated on standing as extremely well formed octahedra. The product was homogeneous and melted sharply with decomposition at 250–251° (bath at 200° and temperature slowly raised). Recrystallisation from water caused no alteration either of crystalline form or of melting point. The salt was anhydrous. Analysis of this salt gave the following results:

0·1043 g.; 0·0300 g. Pt. Pt = 28·76.
0·1071 g.; 0·0312 g. Pt. Pt = 29·13.
0·1384 g.; 0·0401 g. Pt. Pt = 28·97.

*0·1641 g.; 0·1114 g. CO_2; 0·0654 H_2O; C = 18·5; H = 4·4.
0·1320 g.; 0·0846 g. CO_2; 0·0475 H_2O; C = 17·5; H = 4·0.

*0·1346 g.; 9·0 cc. N_2 (moist) at 766 mm. and 11°. N = 8·0.
0·1184 g.; 8·5 ,, ,, 753 ,, 13°. N = 8·38.

Calculated for $[C_5H_{13}O_2N_2]_2PtCl_6$. Pt = 28·93; C = 17·80; H = 3·86; N = 8·31.

The analysis of this salt for C, H, and N gave some difficulty since NO was readily evolved. Unless special precautions were taken and doubly long reduced copper or silver spirals employed some of the oxides of nitrogen escaped reduction and results were obtained such as those instanced above and marked with an asterisk. In such cases nitrous acid was readily shown to be present in the potash bulbs or nitrometer.

The platinichloride was converted into the aurichloride by decomposing the former by means of excess of potassium chloride, evaporating the solution to dryness, extracting with alcohol, evaporating off the alcohol and precipitating the concentrated aqueous solution of the residue with an aqueous solution of gold chloride. The aurichloride thus formed was recrystallised from water, in which it is moderately soluble, and was obtained in the form of plates which melted with decomposition at 256° after sintering from about 200°.

Analysis:

0·1753 g.; 0·0727 g. Au; Au = 41·47.

Calculated for $[C_5H_{13}O_2N_2]AuCl_4$. Au = 41·73.

Hydrolysis of pseudo-muscarine. 0·4 gram of the platinichloride of pseudo-muscarine was boiled under reflux for 2 hours with 5 cc. of 10% hydrochloric acid. The solution was then concentrated and on standing there was obtained 0·07 gram of a platinichloride crystallising in needles and hexagonal plates, melting at 237–238°.

Analysis gave the following:

0·0646 g.; 0·0199 g. Pt. Pt = 30·8.

Calculated for $[C_5H_{14}ON]_2PtCl_6$. Pt = 31·6 per cent.

The mother liquors from the platinichloride were decomposed by potassium chloride and the aurichloride of the base obtained as described above. This was moderately soluble in hot water and melted at 261–262°.

Analysis:

0·1387 g.; 0·0610 g. Au. Au = 44·0.

Calculated for $[C_5H_{14}ON)AuCl_4$. Au = 44·4 per cent.

The mother liquors from the aurichloride were decomposed by H_2S and on treatment with mercuric chloride gave a crystalline salt melting sharply at 249° which when mixed with choline mercuric chloride showed no alteration of melting point.

On hydrolysis therefore pseudo-muscarine yields choline. The formation of nitrous acid on hydrolysis was readily shown by boiling a solution of the chloride of the base for a few moments with dilute alkali, acidifying the solution, and adding to a solution of potassium iodide and starch. The dark blue colour of the starch-iodine complex at once appeared.

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